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BIOCHEMICAL AND IMMUNOCHEMICAL STUDIES ON TICKS
(IXODIDA: IXODIDAE).

A Thesis
Submitted to the University of London
for the Degree of Doctor of Philosophy
(Faculty of Science)

by

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ABSTRACT

In developing a vaccine against *Rhipicephalus appendiculatus*, an important tick ectoparasite of livestock in Africa, a necessary first step is identification of antigens which give protective immunity. Antigen profiles of extracts of unfed immature and adult *Rh. appendiculatus* ticks and their fractions were compared by immunoblotting with sera raised against tick^{infestation} and against whole or fractionated extracts. Several antigens (51.5, 40, 36.5 and 23kDa) were observed to be absent in extracts of fed or partially fed adult ticks. Antigens of 84, 60 and 40kDa were consistently detected in extract fractions shown in immunisation/tick challenge experiments to be immunogenic. The 60kDa antigen was found both in soluble and membrane fractions, whilst the 84 and 40kDa antigens did not appear to be membrane associated. The 84 and 40kDa species appeared heavily glycosylated with a broad range of carbohydrate moieties being present. The 60kDa antigen did not bind significantly to any of the lectins used, suggesting only minimal glycosylation. Probing extracts of unfed larval ticks of different species with serum raised against an immunogenic fraction of *Rh. appendiculatus* unfed nymphal extract revealed 60kDa antigen species in each of the five different tick species. Immunostaining of sections of unfed adult female *Rh. appendiculatus* illustrated marked differences in the distribution of antigens associated with adult tick feed and those associated with immunisation with extracts of

unfed nymphal ticks and their fractions. Of the unfed immature *Rh. appendiculatus* extract fractions used in immunisation and adult tick challenge feed experiments in guinea pigs, SEHPLC fraction 2 and the 45% ammonium sulphate supernate fraction were found to be the most protective. The prospects for developing an anti-*Rh. appendiculatus* vaccine are discussed, and antigen purification strategies are suggested.

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1. INTRODUCTION AND REVIEW OF LITERATURE.

Ticks are obligate ectoparasites belonging to the Suborder Ixodida, Order Parasitiformes, Subclass Acari of the Class Arachnida. They are haematophag^ous, sucking the blood of a wide range of both warm and cold blooded vertebrates. The Ixodida comprise three families; one of these, the Nut^talliellidae, consists of a single species from southern and south western Africa (Hoogstraal, 1985). There are about 150 species belonging to five genera in the second family, the Argasidae (Varma, 1987); these tough and leathery ticks lack a dorsal plate or scutum and are referred to as 'soft' or argasid ticks. The third family, Ixodidae, or 'hard' ticks have around 650 species (Hoogstraal, 1985) belonging to 13 genera. All ixodid ticks have a scutum, which in adult males covers almost the whole of the dorsal body surface; in adult females, and the larval and nymphal stages, it is much smaller and restricted to the anterior part of the dorsum. The rest of the body wall is highly expandable and is leathery in appearance, thus enabling the tick to consume vast quantities of blood during feeding. The Argasidae are essentially world-wide in distribution, and this is also true of the Ixodidae although they occur more frequently in temperate regions and can be found in all climatic zones (Balashov, 1972). Relatively few tick species occur in exceedingly wet or dry areas; *Hyalomma* species are found in arid areas, including deserts, and many tick species are capable of tolerating large variations in both humidity and temperature.

1.1 The Economic Importance of Ticks.

The economic losses associated with ixodid tick infestation of livestock are such that effective tick control measures are essential. Apart from the direct effect of tick infestation, such as loss of blood, general unthriftiness and damage to hides, losses due to tick-borne diseases are considerable. In the case of *Boophilus microplus* in Australia, costs of control together with lost production currently amount to around 100 - 150 million Australian dollars per year (Willadsen and Kemp, 1988). Of a world cattle population currently estimated in excess of 1.27 billion, as many as 80% are exposed to the risk of tick infestation. Steelman (1976) estimated annual losses of around 8 billion (8×10^9) US dollars to the cattle industry worldwide; this being the result of direct parasitisation by ticks, transmission of microorganisms and the development of secondary bacterial infection at attachment sites. In many of the developing countries of tropical Africa, tick infestation and tick-borne diseases constitute major hindrances to effective livestock farming. Control of ticks is therefore of great importance in the economy of countries with a livestock industry.

1.2 Medical and Veterinary Importance of Ticks.

Next to mosquitos, ixodid ticks are the most important vectors of human and animal pathogens (Balashov, 1972; Bram, 1975). The number and variety of tick-borne infections afflicting both wild

and domestic animals throughout the world appear to be on the increase (Hoogstraal, 1981). Whilst the epidemiology of some diseases is changing, others have been only recently defined.

A condition referred to as tick paralysis can occur in many farm and wild animals, pets and also in man; the paralysis being brought about by injection of neurotoxins into the host *via* the female tick's saliva during the feeding period. The manifestations of tick toxicosis caused by *Rhipicephalus appendiculatus* include a general loss of condition and productivity in cattle, and a marked decrease in resistance to other infections. In the summer months dense populations of *Rh. appendiculatus* adults may build up. Such heavy populations can cause major tissue damage and may lead to the formation of abscesses or even crumpling, and sometimes loss, of the ear pinna and damage to the udders and tail (Kettle, 1984).

Transmission of arboviruses by tick bite is common, and transovarial transmission is a frequent occurrence. Numerous arboviruses have been associated with ixodid ticks, and most of these can affect both man and a variety of wild and domestic animals; Nairobi sheep disease is transmitted by *Rh. appendiculatus*. The virus appears to be restricted to sheep and goats, in which mortality may be as high as 90%; human infections have been known to occur but are rare and the disease is mild (Kettle, 1984).

Various bacteria have been associated with ixodid ticks; of these the Rickettsiales (mostly gram-negative rod shaped cocci; Krieg and Holt, 1984) are the most important (Kettle, 1984). *Rh. appendiculatus* is a vector of *Rickettsia conori* which causes African tick typhus (also known as Marseilles fever, fièvre bouttonneuse or tick-bite fever) (Scott, 1978).

Of the protozoal diseases transmitted by ticks, Babesiosis and Theileriosis are the most important among cattle (Kettle, 1984). *Rh. appendiculatus* has been found to be a vector of *Babesia bigemina* (Red Water) which can cause fever, anaemia and haematuria in cattle (Walker *et al.*, 1978). Theileriosis is widespread in cattle, and of the five species of *Theileria* found in Africa south of the Sahara, the two forms of *T. parva* (*T. parva parva* and *T. parva lawrencei*) are transmitted by *Rh. appendiculatus*. *T. p. parva*, causes East Coast fever in cattle in eastern and central Africa, and *T. p. lawrencei* causes corridor or buffalo disease (Young *et al.*, 1988). Among susceptible cattle morbidity may be 100% and mortality may be high (Kettle, 1984). The principal symptoms are enlargement of the lymph nodes adjacent to the feeding lesion, the presence of schizonts in the lymph nodes, spleen and liver, and toxic effects on the bone marrow.

1.3 The Tick Life Cycle.

A thorough knowledge of the tick life cycle and biology is essential to our understanding of their effect on host animals.

The argasid and ixodid life cycles exhibit incomplete metamorphosis and involve a larval and one or more nymphal stages. The eggs hatch to produce six-legged larvae, which after taking a bloodmeal moult to produce eight-legged nymphs. Following feeding the nymphs develop into adults. The argasids are rapid feeders and engorge in under one hour. They usually have several (four to seven) nymphal instars each of which require a blood-meal before they can progress to the next instar (Varma, 1987). The studies presented here are concerned almost exclusively with the ixodid tick *Rh. appendiculatus* (the brown ear tick) and therefore any subsequent references to argasid ticks will be kept to a minimum.

To obtain a full bloodmeal ixodid ticks remain attached to their hosts for long periods, often lasting 1 - 4 weeks, depending on the stage; the adults take longer to engorge than the immatures. Upon cessation of feeding the engorged tick drops from the host and seeks shelter in and under ground foliage to develop into the next stage or to lay eggs. The onset of egg laying generally begins as early as 3 - 6 days after completion of feeding. Several thousand small spherical eggs are deposited in a mass in front of the mouthparts (Walker *et al.*, 1978). Once oviposition is complete (as short as 10 days or as long as five weeks) the

ixodid female dies, and 2 - 3 weeks to several months later the eggs hatch into six-legged larvae (0.5 - 1.5mm long). Following several days of inactivity these 'seed' ticks will climb to the tips of grass or other vegetation and 'quest' for a host; on encountering a suitable host they climb aboard and crawl to their preferred attachment sites, these being stage, species and host dependent (Walker *et al.*, 1978).

About 3 - 7 days after attachment the engorged larvae drop to the ground and moult into nymphs. The eight-legged nymphs will seek out a host in a manner similar to larvae, attach themselves to their chosen sites and initiate feeding. The fully engorged nymphs detach and fall to the ground after 5 - 10 days of feeding and moult into males or females. Unlike the Argasidae, ixodid ticks have only one nymphal stage. Following a period of minimal activity, lasting around seven days, the adults climb vegetation and quest for a host. Female ixodids take as much as 400 times their own body weight of blood and remain attached to their hosts for 1 - 4 weeks. Ixodid males, in comparison, take significantly smaller bloodmeals. When feeding ceases the adult ticks fall to the ground and seek refuge under the surface-vegetation.

Most ixodid species are not essentially host specific; *Boophilus* ticks feed principally on cattle, but many of the medically important species exhibit less specificity and will feed on a wide variety of mammals, specificity often being a matter of

encounter. Larvae and nymphs often prefer to feed on small mammals or birds, whereas adults tend to have a predilection for larger mammals, such as cattle, sheep and horses. All life stages of ixodid ticks may feed on man, adult ticks less so than the younger stages.

A lack of suitable hosts, but more often climatic conditions, may prolong the tick's life cycle by months or sometimes years. Development and breeding may continue throughout the year, with seasonal fluctuations, in warm countries; whilst development in temperate regions may halt temporarily or be prolonged during the winter months.

The majority of ticks follow a three-host life cycle like that mentioned, however a number of ticks have a two-host life cycle, and others such as *Boophilus* species, feed and moult from larva through nymph to adult all on the same host (Varma, 1987).

Rh. appendiculatus is a three-host tick. The adult female may lay up to 5700 eggs, which in the summer hatch within three weeks, but in winter may take up to three months (Walker *et al.*, 1978). The larvae feed mainly around the head of the host; particularly on the cheeks, muzzle, around the eyes and the outer ear flap. The nymphal instars of *Rh. appendiculatus* have a predilection for the areas around the base of the ears, back of the poll and neck top-line, and many are found attached to the ear flap or pinna. Over 50% (can be up to 90%, Newson 1978) of

Rh. appendiculatus adults feed around the ear pinnae, and with a heavier infestation they spread around the eyes, to the top-line of the neck and the back of poll, the perineum and the ventral surface, and in a very heavy infestation to almost anywhere on the body (Walker *et al.*, 1978). *Rh. appendiculatus* has an extremely wide host range. It is commonest on cattle and may be present in enormous numbers, with all stages feeding readily. Other livestock such as sheep are also frequently infested and numerous wild animals can act as hosts; of these, wild antelope and buffalo are especially favoured. Quite large numbers of immature *Rh. appendiculatus* have been found on mongooses and suricates, and hares have also been recorded as hosts as have many rodent species (Walker *et al.*, 1978). *Rh. appendiculatus* is found in most of eastern, central and southern Africa. In southern Africa (Malawi, S. Africa, Zambia and Zimbabwe) the adults are mainly active in the summer (rainy season, October/November to February/March); larvae in late summer, autumn and early winter (March/April to June/July); and nymphs in autumn, winter and early spring (dry season, April to September), and there is a single generation each year (Walker *et al.*, 1978; Rechav, 1982). Nearer the equator (Uganda, Tanzania and Kenya) development rates are increased by higher temperatures and there may be more than one generation *per* year (Newson, 1978; Rechav, 1982).

1.4 Tick Control Methods.

Chemical control of ticks, in tandem with immunisation against tick-borne disease or chemotherapy, is still the most common method for controlling ticks and tick-borne diseases. Despite the high costs involved in their use and of training personnel, tick control has been almost totally dependent on such chemicals (Wharton, 1976). In commercial farming costs are not a great problem, but in a country such as Zambia, where out of 2.4 million cattle only 350 000 are in the commercial sector, the high costs of acaricides are prohibitive. The financial resources of the traditional small pastoral herdsman are nowhere near the levels required for the purchase of chemicals let alone the costs of maintaining acaricidal sprays and dip tanks. In addition, many ticks have developed resistance to acaricides. It is in the genus *Boophilus* that the greatest levels of acaricide resistance have been observed; but resistance in multiple host ticks including *Rh. appendiculatus* has also been reported (Wharton, 1976; Baker, 1978; Solomon, 1983; Luguru *et al.*, 1987; Willadsen and Kemp, 1988). Resistance has been reported to almost all tick control chemicals (Solomon, 1983). Durand (1976) warned that the projected economic return before the development of resistance was insufficient, when compared to the high cost of developing new chemical compounds, to stimulate the production of new acaricides.

The development of tick resistance to acaricides and the high costs associated with these chemicals has stimulated interest in alternative non-chemical methods of control, such as natural (innate) and acquired resistance to ticks (Utech *et al.*, 1978). Cattle exhibiting innate or acquired resistance to ticks cause significant reductions in tick yield, engorgement weight (Utech *et al.*, 1978) and reproductive capacity (Roberts and Kerr, 1976). This results in fewer ticks being carried over to the next generation; thus there are great epidemiological and economic implications. Manipulation of the host immune response to ticks would provide a novel control strategy of considerable benefit worldwide. Trager (1939 a and b) investigated host immunity to ticks in the laboratory, but only limited interest (Kelley, 1943; Bonsma, 1944) was taken in the subject until the late 1960's. Balashov (1972) discussed briefly some of the early investigations (to 1967), and Nelson *et al.* (1977) included some of the work on ticks in a review of ectoparasites. In recent years the upsurge in interest has brought forth an abundance of publications on the subject, which has in turn led to several review articles (Willadsen, 1980 and 1986; Wikel, 1982 and 1984a; Brown, 1985 and 1988c; Wikel and Whelan, 1986; Willadsen and Kemp, 1988). A selected bibliography covering material to early 1980 was produced by McGowan and Barker (1980).

1.5 Host Resistance to Ticks.

It was Johnston and Bancroft (1918) who first observed that *Bos indicus* cattle exhibited levels of innate resistance to ticks, but until the conclusive demonstration of acquisition of resistance by *Bos indicus* (Zebu type) (Wagland, 1975 and 1978) and by *Bos taurus* (European type) (Roberts, 1968 a,b and c) to *Bo. microplus*, many scientists remained unconvinced that host resistance to ticks really existed. It soon became apparent that significant differences occurred between discrete breeds of cattle in their ability to acquire meaningful levels of resistance to *Bo. microplus*; with the exception of Jersey cattle, *B. taurus* were inferior to *B. indicus* breeds and their crosses (Sutherst and Utech, 1980). It was not until the early 1970's that Wharton *et al.*, (1970) and Hewetson (1972) indicated that the ability to acquire a worthwhile degree of resistance was heritable. It was also suggested that such an ability was dependent upon tick density (Sutherst, Utech, Dallwitz and Kerr, 1973).

The expression of resistance by cattle to *Bo. microplus* becomes apparent during the first 24 - 48 hours of larval attachment (Roberts, 1968c). Kemp *et al.* (1976) demonstrated that during this period, detachment and re-attachment of larvae occurred more frequently on resistant hosts than it did on naive or susceptible hosts; consequently greater numbers of larvae on resistant hosts either failed to gain weight or lost weight and subsequently died, than did larvae feeding on naive or susceptible hosts.

This also appears to be true for *Rh. appendiculatus* larvae and nymphs (Tatchell, 1986), and could be expected to hold for other tick species as well. Naive *B. indicus* x *B. taurus* crossbreeds appear to carry more ticks than naive pure *B. indicus* breeds but dramatically less than naive pure *B. taurus* breeds (Latif, 1984).

Tatchell and Easton (1986) and Kaiser *et al.* (1982) have demonstrated the limiting effect of host resistance mechanisms on tick numbers in cattle. Comparisons of high resistance and low resistance groups of cattle indicated that the ranking of resistance was constant for all instars and species of ticks and in terms of tick burdens such rankings were highly repeatable. It was concluded that by culling or by selectively breeding herds of zebu cattle, their resistance could be increased, thereby enhancing tick control as a whole. However, Kaiser *et al.* (1982) observed that cattle took longer to acquire resistance to *Rh. appendiculatus* than to other tick species. This species is regarded as the most serious pest tick affecting cattle as it is the only natural vector of *T. parva* (East Coast Fever, ECF). The inadequate stimulation of host resistance by *Rh. appendiculatus* indicates that this tick will remain in greater need of control than the other tick species parasitising cattle in Africa (Tatchell, 1986).

Although the potential of exploiting highly resistant hosts has been amply demonstrated (Wharton and Norris, 1980; Utech and Wharton, 1982; Sutherst, 1983), a greater understanding of the

mechanisms of host resistance is required before it becomes practical to apply such immunological knowledge in this manner.

1.6 Host Resistance Mechanisms.

Resistant hosts affect tick feeding and reproduction in a variety of ways: successful attachment may be prevented; there may be death *in situ*; the bloodmeal may be smaller thereby reducing the engorged weight; there may be a reduction or abolition of egg-laying; the egg hatch rate may be reduced or there may be diminished moulting success of immature stages. Immune expression depends on the recognition of target antigens and subsequent damage to the ticks. However, the immune system of cattle, the most important livestock host of ticks, is poorly understood, and because of the prohibitive size and cost of cattle, much of the knowledge of the immune resistance response to ticks and the components involved has been acquired by the use of laboratory animal hosts (mainly guinea pigs and rabbits) as model systems. However, convenient as this may be, it is important to remember that results in laboratory animals do not necessarily give an accurate indication of what occurs in a natural host/tick relationship. For example Randolph (1979) observed that whilst the natural host of *Ix. trianguliceps*, the long-tailed field mouse (*Apodemus sylvaticus*), develops no resistance to the tick, laboratory mice readily do. Norval (1978) observed that sheep (natural host) and rabbits (laboratory model) appear unable to acquire resistance to *Am. hebraeum*

larvae and nymphs, and in a recent report the same observation was made for sheep exposed to repeated infestations of *Am. hebraeum* adults (Norval *et al.*, 1988). Chabaud (1950) observed a similar phenomenon when any stage of *Rh. sanguineus* was fed on dogs, whilst guinea pigs rapidly acquired resistance and rabbits showed a response intermediate to those of dogs and guinea pigs. Guinea pigs rapidly acquire long-lasting resistance to feeding by ticks (Garin and Grabarev, 1972; Allen, 1973; Bagnall and Rothwell, 1974; Brown and Knapp, 1981; Heller-Haupt *et al.*, 1981; Brown, 1982; Askenase *et al.*, 1982). With a one-host tick such as *Bo. microplus* there is no alternative model and the natural host (cattle) must be used.

Passive serum transfer from sensitised guinea pigs to naive recipients has been found to induce varying degrees of protection against tick challenge (Roberts and Kerr, 1976; Brossard, 1977; Fujisaki, 1978; Mishaeva *et al.*, 1981; Askenase *et al.*, 1982). Wikel and Allen (1976a) found serum transfer to be ineffective with *De. andersoni*; they did however, observe loss of immunity when B cells were specifically suppressed with cyclophosphamide (Wikel and Allen, 1976b), suggesting that antibody does play a role. Graziano and Askenase (1979) have shown that, in the case of *Am. americanum*, the active component in immune serum is the antibody IgG₁. Heat treatment of serum had no apparent effect on antitick activity so IgE was probably not involved (Graziano *et al.*, 1981). Removal of IgG₁ from whole immune serum destroyed the ability of the serum to confer tick resistance upon tick-

naive hosts. Only a weak cutaneous basophil response was observed and there was no macroscopic erythema. Upon transfer of purified IgG₁ from immune hosts, a high level of resistance was observed; there was a strong basophil response and clear evidence of macroscopic erythema. Recently published work by Worms *et al.* (1988) indicates the requirement of perhaps IgE or IgG₂ in addition to IgG₁ in the expression of guinea pig resistance to *Rh. appendiculatus* larvae.

Complement appears to play a major role in acquired resistance to ticks (Wikel and Allen, 1977). Salivary gland antigens, complement and IgG were all found deposited at the dermo-epidermal junction at the attachment site of *De. andersoni* larvae on resistant guinea pigs (Allen *et al.*, 1979). It has been suggested by these authors that the local inflammatory lesion may involve and originate from antigen-antibody interaction and complement activation. Wikel and Allen (1977) used cobra venom factor to deplete tick-resistant guinea pigs of the C3 component of the complement system; this led to greatly impaired levels of immune expression to *De. andersoni* larvae. In a recent paper Papatheodorou and Brossard (1987) reported an eight fold increase in C3 levels in rabbits exposed to a third infestation of *Ix. ricinus* adults when compared to C3 levels in unexposed rabbits. It is suggested that the C3 component is particularly important in the response of higher vertebrates to acute inflammation and tissue damage (Hartweit *et al.*, 1973).

Brown and Askenase (1981) and Askenase *et al.* (1982) have successfully transferred immunity using peritoneal exudate cells, and others have been successful using lymph node cells (Bagnall, 1975; Wikel and Allen, 1976a); however these transfers have always involved a mixture of lymphocyte and other cell types. Allen (1973) first reported that basophils were the major cell type involved in the inflammatory response accompanying guinea pig resistance to ticks. Basophils are transported to the dermis *via* the blood from the bone marrow in local immunological reactions. High levels of basophils have been observed in guinea pig immune cutaneous resistance responses to: *Am. americanum* (Brown and Knapp, 1981; Brown, 1982); *De. andersoni* (Allen, 1973); *De. variabilis* (Brown, 1985); *Ix. dammini* (Krinsky *et al.*, 1982); *Ix. holocyclus* (Brown, 1985); *Rh. appendiculatus* (Brown *et al.*, 1982b) and *Rh. sanguineus* (Brown and Askenase, 1981). Brown *et al.* (1982b) found that in guinea pigs the dermis is dominated by basophils and eosinophils by day four of a primary challenge, when more than 80% of *Am. americanum* larval ticks will have fed and detached. In a secondary challenge, basophils were found to make up 60 - 70% of the infiltrate by 24 hours post-tick-attachment (Brown and Askenase, 1981; Brown *et al.*, 1982a). Eosinophils (10 - 20% of the infiltrate) were the next most common cell type. At 72 hours post-tick-attachment greater than 90% of basophils appeared degranulated (Brown *et al.*, 1982a). Basophils are the circulating counterparts of mast cells and upon stimulation degranulate to release several mediators involved in vasodilation (histamine), C3 activation (tryptase), eosinophil chemotaxis (ECF) and neutrophil chemotaxis (NCF)

amongst others (Roitt, 1988). Electron microscope studies of *Rh. appendiculatus* feeding sites on actively sensitised guinea pigs indicate that basophils arrive at the sites and then degranulate (McLaren *et al.*, 1983a). At six hours post-attachment only minimal degranulation is observed, but by 12 hours many basophils exhibit anaphylactic degranulation; a peak basophil response appears to occur by 18 hours, and tick-attachment is characterised by sheets of basophils surrounding the tick's mouthparts. The presence of degranulation sacs is an indication of compound exocytosis. Blood eosinophils have been observed to respond to active tick feeding (Brown and Askenase, 1982), guinea pig blood eosinophil levels in a secondary challenge were found to be twice those observed during a primary challenge with *Am. americanum* larvae. This is consistent with a thymus-dependent anamnestic immune response, as in primary hosts, blood eosinophil levels subside after the ticks have fed to repletion and dropped off. Brown *et al.* (1982b) observed that tick-sensitised guinea pigs failed to express resistance after administration of antibasophil serum; in addition although blood and bone-marrow eosinophil levels were increased by tick-challenge, eosinophils were found in reduced numbers at tick feeding sites. This suggests a role for basophils in the recruitment of eosinophils to the reaction sites. Sensitised guinea pigs treated with antieosinophil serum exhibited a level of tick rejection intermediate between primary and secondary infestations. In studies involving guinea pigs and *De. andersoni* adults (Allen *et al.*, 1979; Nithiuthai and Allen, 1984, 1985) macrophages and

epidermal Langerhans cells were shown to be accessories in antigen presentation, capable of inducing lymphocyte proliferation. Paine *et al.* (1983) have shown that histamine and serotonin (5-HT) specifically affect the feeding behaviour of *De. andersoni*; *in vitro* feeding of ticks with serum containing added histamine and serotonin led to an inferred decrease in salivation and feeding activity. Brown and Askenase (1985) observed no impairment of immunity when guinea pigs were treated with the histamine antagonists mepyramine and cimetidine, so the requirement of histamine is somewhat uncertain. Thus, it would appear that the immunity of guinea pigs to ticks requires antibody or lymphocyte-dependent basophil and eosinophil recruitment to the tick feeding site followed by the release of substances that damage the tick. Interestingly, guinea pigs mount an anamnestic basophil response to *Ornithodoros tartakovskyi* and yet they do not become immune (McLaren *et al.*, 1983b). Presumably, these argasid ticks are protected by their ability to feed rapidly. Even when fed on an animal with pre-induced basophilia these argasid ticks may remain unaffected (Askenase and Worms, 1979).

Den Hollander and Allen (1985a) have shown that the main events at tick feeding sites of mice infested with *De. variabilis* larvae are the accumulation and degranulation of mast cells and eosinophils. Work with mast cell deficient strains of mice indicated that mast cells were involved to a small extent in immunity (den Hollander and Allen, 1985b). Investigations by Matsuda *et al.* (1985) suggest a major role for mast cells in the

resistance of mice to *Haemaphysalis longicornis*. When bone marrow cells from normal donors were injected into the mast cell deficient mice, expression of resistance developed, and mast cell numbers were observed to be normal. In another experiment the authors grafted skin from normal donors onto mast cell deficient recipients and repeatedly challenged the graft sites with ticks; the animals expressed resistance. Basophils were not observed in any mice, though the numbers of eosinophils were affected by the presence or absence of mast cells.

Bowessidjaou *et al.* (1977) have shown that repeated infestation of rabbits with *Ix. ricinus* adults leads to an impaired feeding and reproductive performance by the ticks. Transfer of immune serum to naive hosts resulted in a low level immediate type skin sensitivity reaction to *Ix. ricinus* salivary gland extract; both antitick IgG and antitick homocytotropic antibodies were demonstrated in the recipients (Brossard and Girardin, 1979). The accumulation of eosinophils and basophils and degranulation of basophils and mast cells were observed in immune rabbits (Brossard and Fivaz, 1982); degranulation of basophils and mast cells is usually associated with histamine release (Roitt *et al.*, 1985). Additionally, it was shown (Brossard, 1982) that the histamine antagonist mepyramine inhibited the expression of resistance. This is in contrast to Brown and Askenase (1985) who observed no impairment of immunity in guinea pigs treated with mepyramine or cimetidine, another histamine antagonist. High numbers of basophils were observed at feeding sites on rabbits

expressing resistance to *Ix. dammini* (Krinsky *et al.*, 1982) and Gill and Walker (1985), looking at adult *Hy. anatolicum* feeding sites on tick-resistant rabbits, reported significant increases in basophil numbers (9% of cells) when compared to basophils at feeding sites on tick-susceptible rabbits (3% of cells); however, neutrophils (upto 68% of cells in tick susceptible, and 53% of cells in tick resistant rabbits) appeared to be the dominant cell type. Eosinophils represented upto 10% of the cells in tick susceptible rabbits and 21% of cells in tick resistant animals. Rubare-Akiiki and Mutinga (1980), working with *Rh. appendiculatus*, observed only minimal cutaneous basophilia in tick resistant and naive rabbits, and in a more recent report, Manyasi (1987) noted similar observations in rabbits infested with adults of *Rh. appendiculatus* or *Am. variegatum*, in addition, mast cells appeared to be absent from the attachment site, and eosinophils predominated.

Without doubt, the most thoroughly studied tick-host relationship involving a domestic animal is the bovine response to the economically important one-host tick *Bo. microplus*. Cattle resistant to *Bo. microplus* exhibited accumulations of lymphocytes and eosinophils at attachment sites together with inflated circulatory histamine levels (Riek, 1962). The resistance which cattle acquire to this tick may result in the rejection of almost all (>99%) the larvae which initially attach. This involves an immediate hypersensitivity reaction. When antigens isolated from *Bo. microplus* larvae are injected intradermally into resistant cattle, the hosts' reactions correlate well with their resistance

to ticks (Willadsen and Williams, 1976; Willadsen *et al.*, 1978). Mast cell concentration and degranulation, eosinophil accumulation and degranulation and total skin histamine are all components of immediate hypersensitivity reactions and have all been correlated to resistance (Schleger *et al.*, 1976; Willadsen *et al.*, 1979). Kemp and Bourne (1980) have shown that tick detachment from bovine skin is specifically induced by histamine, and Tatchell and Bennett (1969) were able to block resistance in sensitised cattle with antihistamine. It has been suggested that histamine originally released by mast cells is accumulated and transported by eosinophils (Schleger *et al.*, 1981). Mast cells have been observed at *Ix. ricinus* attachment sites on cattle (Pavlovskii and Alfrevva, 1941), and a cutaneous basophil hypersensitivity (delayed type) response was stimulated by *Ix. holocyclus* infestation of sensitised *Bos taurus* (Allen *et al.*, 1977). Binta *et al.* (1984a) have shown that histamine-dependent skin reactions are produced by cattle resistant to *Rh. appendiculatus* upon injection of allergenic larval tick extracts; and that such reactions are transferred with bovine homocytotropic antibody (Binta *et al.*, 1984b). Work by Manyasi (1987) indicated that eosinophils are the major cell type involved in the inflammatory response accompanying resistance of cattle to *Rh. appendiculatus*. In addition to the predominance of eosinophils, neutrophils (heterophils) and macrophages were consistently present in tick-feeding lesions on resistant cattle. Both cellular and serum immunoglobulins were detected in inflamed tissue, but only minimal basophilia was observed.

It is clear from the above that there are two components involved in host resistance to ticks; a cell-mediated component involving a delayed type cutaneous inflammatory response associated with a range of cell-types, being dominated by basophils in guinea pigs and eosinophils in larger mammals such as rabbits and cattle; and a humoral component involving IgG₁ and probably several other antibody types associated with an immediate type hypersensitivity, stimulation of T and B lymphocytes and involvement of the classical complement pathway. Successful immune expression appears to depend upon the effective interaction of these two immune components.

1.7 Immunosuppression.

As a means of maximising the chances of survival, many parasites induce host immunosuppression as a means of eluding the host's immune responses (Ogilvie and Wilson, 1976).

Reich and Zorzopulos (1980) observed a decline with time of host antibody responses to *Bo. microplus* larval phosphomonoesterase, and speculated that this decline involved some form of immunosuppressive mechanism, and a proteolytic-enzyme inhibitor isolated from *Bo. microplus* salivary glands was shown to block the action of complement (Willadsen and Riding, 1980). Work by Wikel (1976) and Wikel and Osburn (1982) has suggested impairment of host immune competence by repeated tick infestation. In a recent paper Wikel (1985) has shown that direct haemolytic

plaque-forming cell responses to a thymic dependent antigen were reduced in guinea pigs with *De. andersoni* larval infestations. The indirect plaque-forming response to sheep red blood cells was reduced towards the end of a primary infestation; but during a secondary infestation the ability to mount such a response was inhibited to a much greater extent. This tick-induced immunosuppression disappeared within four days of cessation of feeding (secondary infestation). Thus despite the host being resistant, immune competence was still impaired. Ribiero *et al.* (1985) suggested that the immunosuppressive characteristics of *Ix. dammini* saliva may be due to the presence of a prostaglandin, PGE₂.

Callow and Stewart (1978) observed that cattle infected with the piroplasm *Babesia bovis* exhibited reduced levels of immunity to its natural tick vector *Bo. microplus*. Heller-Haupt *et al.* (1983) showed that immune expression to *Rh. appendiculatus* larvae, nymphs and adults was blocked in rabbits infected with *Trypanosoma congolense*. Thus it appears that parasitic infections of the host, whether tick-borne or not, are an important additional parameter affecting host immunity to ticks.

1.8 Effects of Host Immunity on Pathogen Transmission.

Several authors have demonstrated reduced transmission of tick-borne pathogens in tick-resistant hosts. Bell *et al.* (1979) showed that animals expressing resistance to ticks exhibit

increased resistance to the tick-borne bacterium *Francisella tularensis*. Jones and Nuttall (in press) fed Thogoto virus infected *Rh. appendiculatus* adults (donors) alongside uninfected *Rh. appendiculatus* nymphs (recipients) on guinea pigs resistant or naive to ticks. On resistant guinea pigs, there was a significant reduction in the number of recipient ticks acquiring the virus. Protection against tick-borne encephalitis virus was observed in animals that had received antiserum to tick salivary antigens (Votyakov and Mishaeva, 1980); thus, it is likely that immunisation against ticks may not only protect against tick infestation but may also reduce the chances of disease transmission.

1.9 Cross Resistance.

Reports of both intrageneric (interspecific) and intergeneric cross-resistance have been plentiful. Brown and Askenase (1981) observed significant levels of resistance when *Am. americanum* sensitised-hosts were challenged by *Rh. sanguineus*; the reverse was also found to occur. Heller-Haupt *et al.* (1981) reported low levels of cross-resistance between *Am. hebraeum* and *Am. variegatum*, but not between *Am. hebraeum* and *Rh. appendiculatus*. Trager (1939b) observed cross-resistance between *De. variabilis* and *De. andersoni*. Work by McTier *et al.* (1981) confirmed Trager's observation and also demonstrated cross-resistance between *Am. americanum* and *De. variabilis* on guinea pigs. Such reports of cross-resistance are indicative of common antigens

both between species and between certain genera. However, despite the poor feeding performance of *Am. americanum* on *Rh. sanguineus* resistant hosts, transfer of immune serum from *Rh. sanguineus* sensitised hosts to naive animals failed to induce resistance to *Am. americanum* (Brown and Askenase, 1981); but serum from hosts exposed to *Ix. dammini* was found to be effective against *Am. americanum*.

1.10 Immunisation Against Ticks.

Trager (1939a) was the first, nearly 50 years ago, to demonstrate the possibility of vaccinating against ticks; but it was the work of Allen and Humphreys (1979) which made host vaccination a practical alternative to chemical control of ticks. The authors stimulated interest in the subject by showing that ticks fed on guinea pigs immunised with extracts of partially fed *De. andersoni* females were unable to engorge fully. In addition to greatly reduced engorgement weights, there was reduction or abolition of egg-laying, and those eggs that were laid were non-viable. When the authors vaccinated cattle with an extract of reproductive organs and midgut, although the number of ticks engorging was not affected, both engorgement weight and egg laying were significantly reduced.

A variety of antigen sources have been used in attempts to vaccinate animals against ticks. These have ranged from extracts of whole fed or unfed adults or unfed immature ticks, to

haemolymph, gut tissue, cement (salivary gland derived material that solidifies around the mouthparts during feeding to aid in anchoring the tick to the host) and salivary glands.

It is salivary gland antigens upon which most attention has been focussed; not surprisingly perhaps, as the gland begins to secrete early in attachment and contains several enzymes involved in mouthpart penetration (Brown 1985). Salivary glands for use in vaccination experiments are removed from actively feeding females. Köhler *et al.* (1967) were able to induce resistance to *Hy. anatolicum excavatum* by vaccination of rabbits with extracts of salivary glands and Garin and Grabarev (1972) used salivary glands successfully with *Rh. sanguineus*. Other workers (Brossard, 1976; Wikel, 1976, 1981; Brown *et al.*, 1984) have used salivary glands from a variety of tick species to immunise guinea pigs, rabbits and cattle. Brown *et al.* (1984) showed it was possible to induce some degree of tick rejection by immunisation with salivary gland derived cement material. In a recent paper, Ben-Yakir and Barker (1987) used cell-free haemolymph from *Am. americanum* and *De. variabilis* to immunise rabbits; high antibody titres (10^5) were achieved, but mean tick engorgement weights and weights of egg masses were not significantly different from non immunised control animals.

Several workers have obtained good results using extracts of whole fed female ticks (Allen and Humphreys, 1979; Johnston *et al.*, 1986). Indeed Johnston *et al.* claim levels of resistance to

Bo. microplus far in excess of that achieved following natural infestation. Mongi *et al.* (1986b) obtained significant reductions in engorged weight of *Rh. appendiculatus* females fed on rabbits immunised with fed female extracts; however the rabbits had been vaccinated one year previously with extracts of unfed female ticks and then challenged by a larval feed; in addition the total number of rabbits used was only two, too small to be conclusive. As little work has been done on the duration of immunity, it is uncertain whether or not the two rabbits would have still exhibited levels of immunity one year later; but work by Stone *et al.* (1983) indicated that levels of immunity to *Ix. holocyclus* persisted in dogs after exposure to three successive infestations of adult ticks for in excess of 53 weeks in beagles and 102 weeks in foxhounds. Willadsen and co-workers have shown that vaccination with crude extracts of partially fed *Bo. microplus* females provides immunity for a minimum of four months (Miller, 1986). Calves vaccinated with extracts of adult *Am. americanum* exhibited resistance upon adult tick challenge feed (McGowan *et al.*, 1981), and extracts of unfed male *Am. maculatum* induced resistance to adult ticks in rabbits (McGowan *et al.*, 1980). Binta *et al.* (1985) attempted vaccination of rabbits with fractionated extracts of unfed *Rh. appendiculatus* larvae, but there was no reduction in engorged weights or egg viability of female *Rh. appendiculatus* fed on vaccinated animals. This is in contrast to results obtained in our laboratory, where extracts of unfed *Rh. appendiculatus* larvae (Heller-Haupt and Varma, personal communication) or nymphs (Heller-Haupt *et al.*, 1988)

have successfully protected guinea pigs against adult tick feed (evidence of interstadial immunity) . Extracts of unfed *Am. variegatum* larvae have been used successfully to induce resistance in guinea pigs to larval tick feed (Heller-Haupt *et al.*, 1987), and extracts of unfed *Am. variegatum* and *Am. hebraeum* nymphs were able to induce resistance in rabbits and guinea pigs to adult ticks of the same species (Heller-Haupt and Varma, personal communication).

Ackerman *et al.* (1980) observed that hosts immunised with midgut extracts of partially engorged *De. variabilis* developed artificial immunity associated with reduced engorged weight of females and reduced fecundity. Recently Wikel *et al.* (1987) successfully immunised guinea pigs with *Am. americanum* gut-derived antigens and Opdebeeck *et al.* (1988a) observed major reductions in fed female weight and egg production (>90%) when *Bo. microplus* ticks were fed on cattle vaccinated with extracts of gut tissue from actively feeding females.

1.11 Isolation and Characterisation of Antigens Involved in Tick Immunity.

Because of the complexity of the material used to induce immunity, isolation of the antigen(s) is essential. Removal of irrelevant proteins and non-immunogenic material as well as potential immunosuppressant molecules is a difficult and time consuming task. To date, practically all work on vaccination has involved crude extracts of whole ticks, salivary glands or

various internal organs, and despite the large volume of publications on antigen characterisation and purification, surprisingly little progress has been made in isolating reactive proteins. Perhaps progress has been inhibited by an incredibly naive view of the complexity of material used to induce immunity. For example, Wikel and Osburn (1982) claimed only eight proteins were present in *De. andersoni* salivary gland extracts, and McGowan *et al.* (1980) estimated that a homogenate of male *Am. maculatum* contained only 22-24 proteins. If one appreciates that several hundred different proteins have been identified in the bacterium *Escherichia coli* (Neidhardt, 1987), then it becomes absurd to believe that so few proteins are present in tick extracts. Such naivety has led to much confusion, with some authors repeatedly contradicting themselves (eg. Brown, see below), hindering both their own work and the progress of others.

Much of the research directed towards antigen characterisation has focussed on antigens associated with the salivary glands. Geczy *et al.* (1971) identified esterases and a carbohydrate splitting enzyme in the saliva of *Bo. microplus*; of these a 30kDa esterase elicited strong immediate hypersensitivity responses in tick sensitised cattle. Willadsen and Williams (1976) isolated a 60kDa esterase or carboxylic ester hydrolase from *Bo. microplus* larvae; and in another paper Willadsen and Riding (1979) reported the presence of a proteolytic enzyme inhibitor in *Bo. microplus* larval extracts. Both these antigens were shown to evoke immediate hypersensitivity reactions upon intradermal inoculation

of resistant cattle (Willadsen and Kemp, 1988). The antigens appeared to be present for prolonged periods at tick attachment sites (Willadsen and McKenna, 1983) and it was shown that esterases are quickly introduced into host skin after tick attachment, and are not found in larvae after feeding (Tracey-Patte, 1980). However, these antigens have not been successfully used to induce immunity (Willadsen and Kemp, 1988). It has been reported that antienzyme antibodies developed against larval phosphomonoesterases after cattle had been infested with *Bo. microplus* larvae (Reich and Zorzopulos, 1980). Willadsen *et al.* (1988) used detergent extraction, gel-filtration chromatography and preparative isoelectric focussing to partially purify antigenic material from extracts of partially fed *Bo. microplus* females capable of eliciting a protective immunological response to *Bo. microplus* in cattle. Isoelectric focussing studies showed that the antigenic material focussed at between pH 5.05 and 5.65, and by gel-filtration the molecular weight of the antigenic fractions was estimated at between 79 and 205kDa. Willadsen, Kemp and co-workers have filed two patent applications, and Opdebeeck *et al.* one, all relating to *Bo. microplus* antigens. As a consequence little has been published with regard to purification and identification of antigens. An article by Miller (1986) inferred that the Willadsen group had isolated 10 antigens from tick gut, and had spent in excess of 10 million Australian Dollars since 1978. Willadsen and Kemp (1988) reported the isolation of a protective antigen present as a minor component of gut membrane. From a starting material of 1.2kg (50 000 partially engorged female *Bo. microplus*) only about

100µg of the protein was isolated, but this was sufficient to demonstrate the efficacy of immunisation. Work by Opdebeeck *et al.* (1988b) has also shown that the midgut antigens are membrane associated.

Of the multi-host ticks, *Am. americanum* has been the most studied. Brown *et al.* (1984) immunoprecipitated material from *Am. americanum* salivary gland and cement extracts with sera from guinea pigs made immune by larval infestation or by vaccination with salivary gland extracts. Analysis by SDS-polyacrylamide electrophoresis yielded a single 20kDa protein in each case. Subsequently, using both the same technique and the same tick species, Brown and Askenase (1984) immunoprecipitated proteins of 17kDa and 95kDa in addition to the 20kDa protein. The same workers (1986 a and b) fractionated *Am. americanum* salivary gland extract by gel-filtration or ion-exchange chromatography. The fractions containing the 20kDa antigen were identified by immunoprecipitation and SDS-PAGE, and were shown to stimulate active cutaneous anaphylaxis in guinea pigs when inoculated intradermally. In a separate experiment, the authors used immunoaffinity purified IgG₁ obtained from serum from a guinea pig made immune by tick-feed to affinity purify *Am. americanum* salivary gland extract. Assay by immunoprecipitation and SDS-PAGE yielded the 20kDa antigen. When used to immunise guinea pigs this purified material elicited a significant degree of protection upon larval *Am. americanum* challenge feed. There was a 21.4% reduction in average larval tick-weight compared to

larvae fed on naive, non-immunised control guinea pigs. In addition 23.4% of larvae were rejected from hosts immunised with the immunoaffinity purified extract which contrasts with a 38.3% rejection rate from hosts immunised with crude salivary gland extract. These results compare with a tick rejection of 47.2% and a weight reduction of 33.7% when larvae were fed on guinea pigs exposed to a previous infestation with *Am. americanum* larvae (Brown *et al.*, 1984). Immunoblotting studies probing *Am. americanum* female salivary gland extracts (separated by SDS-PAGE) with hyperimmune rabbit serum expressing resistance to *Am. americanum* recognised at least 25 antigens ranging from 10 to 127kDa. The same serum recognised only seven antigen bands in male salivary gland extract. Antigens common to both extracts were at 38, 39 and 40kDa and it was suggested that these proteins play an important role in immunity (Brown, 1986). In addition the 20kDa antigen detected in guinea pig studies was also identified by rabbit serum though it was not present at certain stages of the feeding cycle. In a separate report Brown (1988b) refers to the crucial antigens recognised by rabbit antitick serum being at 39 - 41kDa; these proteins were also observed when salivary gland extracts were probed with serum from rabbits immunised with salivary gland extract. Needham *et al.* (1986) carried out immunoblotting studies to identify antigens in salivary glands of fed and unfed *Am. americanum* females. Serum from rabbits made resistant by several adult tick infestations identified antigens in glands from both unfed and fed ticks at 46 and 60 - 75kDa. In addition an antigen band of >200kDa was observed in glands from unfed ticks. This antigen was not

present in glands from fed ticks, which contained additional bands at 130 and 165kDa. Serum from rabbits immunised with salivary gland extract identified two prominent antigens in glands from fed ticks, at 46 and 75kDa. These results suggest that the 46 and 75kDa antigens play an important role in immunity to *Am. americanum* ticks, and differ markedly from those obtained by Brown and colleagues. These contradictory results for the same tick species, make comparison and evaluation of results a difficult task. In a recent paper Brown (1988a) compared the immunoblot profiles of egg extract, unfed larval extract, unfed nymphal extract and female salivary gland extract of *Am. americanum* ticks when probed with guinea pig antilarval, antinymphal and antiadult *Am. americanum* serum. The results indicate that there are many stage specific antigens, but also several antigens common to various tick stages. Of the shared antigens, one of 38kDa was recognised in egg extracts by antilarval and antinymphal sera, in larval extracts by antilarval and antiadult sera, in nymphal extracts by antilarval serum and in female salivary gland extracts by antiadult serum. These results are difficult to interpret as between them the three sera recognise a 38kDa antigen in all four of the extracts, and yet each individual serum only recognises such an antigen in one or two of the extracts; perhaps this is due to different epitopes being recognised by the various sera. In addition Brown suggests that a 25kDa antigen, identified by antilarval and antiadult sera in female salivary gland extracts maybe a degradation product of the 38kDa. Also, it is suggested that the 25kDa antigen is the

same as the 20kDa species reported earlier (Brown *et al.*, 1984). In a further publication, Brown (1988d) compared *Am. americanum* derived midgut extracts with salivary gland extracts by analysing their immunoblot profiles when probed with antiadult tick sera raised in rabbits and guinea pigs. In excess of 25 antigens were identified in each of the extracts. Rabbit antitick serum revealed eight antigens that were present in the gut extract, but not in the salivary gland extract. This compared with nine unique gut antigens when extracts were probed with guinea pig antiserum. Four of these gut antigens were identified by both rabbit and guinea pig antisera; these were at 16, 180, 220 and 270kDa. Brown suggests that the immune response to *Am. americanum* has two components (Brown and Askenase, 1985), an early component involving secretion of salivary gland antigens, and a later one involving gut antigens.

De. andersoni is another multi-host tick that has been intensively studied. Whelan *et al.* (1984) analysed larval extracts of *De. andersoni* and egg extracts of *Am. americanum* by probing immunoblots with sera from *De. andersoni* adult infested guinea pigs. Some pre-infestation reactivity was observed in both extracts, particularly at around 65kDa. Several components of varying affinity were recognised by post-infestation sera, and a predictable pattern of immunoreactive moieties was observed. Antiadult *De. andersoni* serum recognised antigens in *De. andersoni* larval extracts at 25, 35, 41 and 98kDa; of these only the 41kDa antigen was recognised by antinymphal tick serum. *Am. americanum* egg extracts had components that cross-reacted with

anti*De. andersoni* adult-serum at 93, 98 and 111kDa. Wikel and Whelan (1986) used sera from *Am. americanum* resistant rabbits and purebred Brahman calves to probe immunoblots to detect immunogens in *Am. americanum* egg extracts and *De. andersoni* larval extracts. Four antigens were identified in *Am. americanum* egg extracts when probed with the rabbit antiserum, but none of these components were recognised by the calf antiserum. Interestingly when *De. andersoni* larval extracts were probed with the same calf anti*Am. americanum* serum, components of 25, 35 and 41kDa were observed. These corresponded to bands in *De. andersoni* larval extracts identified by guinea pig anti*De. andersoni* serum. In addition the calf serum also identified material at 60kDa as did the rabbit anti*Am. americanum* serum. Such trends in immunological recognition indicate that there may be some conservation in antigenicity across the genera. Gordon and Allen (1987) used sera from hyperimmune tick resistant rabbits to identify by immunoblotting antigens in extracts of *De. andersoni* salivary glands. A large number of antigens were identified, several of which were also recognised by sera from naive rabbits. It was suggested that these may be cross-reacting antigens similar to those found in other ectoparasites to which the rabbits had been exposed. Den Hollander and Allen (1986) reported that antigens of the widely distributed ear mite *Psoroptes cuniculi* cross-reacted with antigens in *De. andersoni* salivary glands. By preparing salivary gland extracts from *De. andersoni* adults at different stages of the feeding process Gordon and Allen (1987) were able to show that two antigens at 31

and 82kDa were secreted into the host soon after attachment and therefore may be important in tick resistance. The authors also used immunoglobulins purified from hyperimmune tick-resistant rabbit sera in immunoaffinity (IA) isolation of *De. andersoni* salivary gland components. An antigen of 172kDa was observed both in IA purified material and in crude salivary gland extract suggesting that it is a major immunological component of saliva. Two constituents of IA purified material, detected by silver staining of SDS-polyacrylamide gels at 15 and 44kDa, were not detected by immunoblotting of salivary gland extract or IA purified material. Although it is possible that these may represent immunological breakdown products, it is also suggested that they are salivary gland components which lost their antigenicity during the harsh treatments of IA purification, SDS-PAGE or immunoblotting.

Immunoblotting studies using sera from rabbits made hypersensitive to *Hy. anatolicum anatolicum* by adult feed demonstrated the involvement of a large number of salivary proteins in the acquisition of resistance (Gill *et al.*, 1986). Nine antigens were observed in the saliva and 17 in salivary gland extracts from female ticks fed for 96 hrs; molecular weights ranged from 14.4 to 130kDa. Con-A lectin binding studies indicated that 12 of the salivary gland antigens and all of the saliva antigens were glycoprotein in nature, and the majority of antigens were found in different feeding stages (0, 24, 96 and 144 hrs). A 130kDa antigen exhibited acid phosphatase activity and a 96kDa displayed both non-specific esterase and

aminopeptidase activity. These two antigens together with one of 103kDa were isolated from SDS-polyacrylamide gels and used in hypersensitivity tests. All three antigens elicited immediate hypersensitivity reactions upon intradermal inoculation of hypersensitised rabbits. In addition the 96 and 103kDa antigens exhibited strong delayed hypersensitivity reactions.

Köhler *et al.* (1967) reported that nine precipitin lines were observed by immunoelectrophoresis studies when *Hy. a. excavatum* salivary gland extracts were immunophoresed with sera from rabbits immunised with *Hy. a. excavatum* salivary gland extracts. Upto six precipitin bands were observed when salivary gland extracts were immunophoresed with sera from rabbits made resistant by tick infestation; five of these bands overlapped with precipitin lines observed with sera from immunised animals.

Sera from *Ix. ricinus* resistant rabbits and mice were used in immunoblotting studies to identify antigens in whole nymphal and adult *Ix. ricinus* extracts (Rutti *et al.*, 1987). Individual variations in immunological response were apparent, but a 25kDa antigen was regularly observed which was absent from whole larval extracts. Vaccination of rabbits with a fraction enriched with the 25kDa antigen altered the ectoparasite fixation rate and affected both their bloodmeal and subsequent egg production.

The first publication on the characterisation of *Rh. appendiculatus* antigens was that of Mongi *et al.* (1986a). They

immunised rabbits with antigen-antibody complexes formed in immunodiffusion reactions between extracts of fully fed female ticks and serum from a rabbit immunised with both unfed tick homogenate and lyophilised homogenate of fully fed female ticks. Adult tick engorgement weights were not affected, neither was egg mass production, but egg hatch rate and the number of viable larvae were both reduced. Analysis of sera from complex immunised animals, by crossed immunoelectrophoresis with the original fully fed female extracts, resulted in the detection of four to five precipitin arcs. In a separate series of experiments the same workers (Mongi *et al.*, 1986b) immunised rabbits with crude extracts of fully fed *Rh. appendiculatus* females (it should be noted that one year previously these rabbits had been vaccinated with an unfed male tick extract and then challenged by larval feed). Crossed immunoelectrophoresis analysis of the resultant antiserum detected 12 tick antigens, compared with only two antigens when antiadult tick infestation sera were used. Immunoprecipitation of fully fed female extract with serum from immunised rabbits yielded nine antigens on analysis by SDS-PAGE; these were at 82, 85, 88, 92, 94, 98, 130, 140 and 180kDa. The 88 and 92kDa antigens were also observed to be components of tick eggs. Immunoprecipitation of tick extract with antisera from rabbits exhibiting acquired immunity to *Rh. appendiculatus* failed to precipitate any tick antigens. Shapiro *et al.* (1986) compared immunoblot profiles of *Rh. appendiculatus* salivary gland, cement, gut and unfed larval and nymphal extracts. Extracts were probed with serum from guinea pigs after

two sequential infestations of *Rh. appendiculatus* adults. Five antigens were particularly prominent in salivary gland extract; at 16, 20, 35, 58 and 94kDa. Three of these antigens were also detected in gut extract (16, 20 and 35kDa) and most of the antigens identified in salivary gland were also observed in tick cement. Other antigens detected in salivary gland extract were at 25, 28, 46, 77, 88 and 120kDa. Most of the major antigens identified in salivary gland extract were not detected in larval and nymphal extracts, though the antigens at 16, 20 and 35kDa were; in addition larval extracts contained a 68kDa antigen. Shapiro *et al.* (1987) used precipitin arcs from crossed immunoelectrophoresis (CIE) reactions to immunise rabbits against adult tick feed. Rabbits were made resistant to adult *Rh. appendiculatus* by three successive infestations. The resultant antitick serum was then used in double immunodiffusion reactions with a mixture of salivary gland and gut extracts. Two to three precipitin lines were observed. These were cut out and used to immunise a tick naive rabbit, and the resultant antiserum was used in CIE reactions with salivary gland extracts. Five precipitin arcs were observed and these in turn were cut out and used to immunise tick-naive rabbits which were subsequently exposed to adult tick feed. Two of the antigen-antibody complexes were actually found to enhance tick-feeding, and only one of the complexes (arc 3) was found to significantly reduce tick feeding (24.7% reduction in engorged weight), but not to the same extent as when ticks were fed on rabbits that had been previously exposed to adult tick infestation (nearly 80% reduction). The monospecific antiserum raised against arc 3

identified two antigens in salivary gland extract in immunoblotting studies; an intensely staining antigen at 90kDa and a faintly staining antigen at 300kDa. It was suggested that the 300kDa antigen was perhaps a high molecular weight precursor of the 90kDa antigen, or an antigenically related molecule. Immunoblotting studies clearly differentiated the 90kDa antigen from the 88 and 94kDa antigens previously described (Shapiro *et al.*, 1986). This 90kDa antigen was subsequently shown by immunocytochemical studies to be a component of the *Rh. appendiculatus* salivary gland's complex a-, d- and e-granules. It also appeared within the chitinous walls of intercalated ducts, and was observed within the labyrinthine spaces of the type III acini of female salivary glands (Venable *et al.*, 1986).

1.12 My Proposed Scheme of Study.

In many parasite systems it is likely that antigens are common to more than one stage of the life cycle (Rajasekariah *et al.*, 1980). However, Willadsen and Kemp (1988) observed no interstadial protection when they immunised calves with material from an extract of partially fed female *Bo. microplus*; only immunity to adult ticks was observed, no effects on larvae and nymphs were noted. Past work in our laboratory (Heller-Haupt and Varma, personal communication) has indicated that immature stages of *Rh. appendiculatus* provide a good source of material for the isolation of protective antigens, being able to protect laboratory animals against interstadial challenge feed with *Rh.*

appendiculatus. Immunisation with whole extracts of unfed larvae or nymphs resulted in protection of the host against challenge by larvae, nymphs and adults.

With this knowledge in mind it was decided to use unfed immature ticks as an antigen source in my studies. The proposed plan was to crudely fractionate extracts by a variety of different methods and test their immunogenicity in immunisation and tick challenge experiments. Analysis of antigenic components in the different fractions would hopefully identify antigens that might be used as potential vaccine candidates. Antigenic components involved in naturally acquired immunity would be identified and compared to those antigens involved in vaccination induced immunity. Antigens would be further characterised and attempts would be made to localise antigens in tick tissues.

Several pieces of evidence point to the involvement of antibodies in the expression of host immunity^t ticks. Brossard (1976) observed that infestation of cattle with *Bo. microplus* led to a significant increase in serum gamma globulin levels (indicative of an increase in antibody production). Similar findings were described by Rechav (1987); he showed a positive correlation between the total number of ticks (several species) on cattle and serum gamma globulin levels. As previously mentioned (see section 1.6) passive serum transfer has been found to confer hosts with the ability to resist tick challenge. In Australia, the development of immunity after vaccination with tick material

has been shown to involve antibodies. Tracey-Patte *et al.* (1986) detected antibody-induced damage to the gut wall in *Bo. microplus*; and Opdebeeck *et al.* (1988) found that antibody titres of sera from vaccinated animals were positively related to host immunity, though there was no correlation between titres and the actual degree of immunity. Gill and Luckins (1987) have recently reported a direct correlation between the degree of resistance to *Hy. a. anatolicum* and IgG antibody titres following successive tick infestations. The implication is therefore, that vaccination induced protective immunity to ticks involves an antibody-mediated component (presumably together with a cell-mediated component). It is with this evidence in mind that my studies have concentrated heavily on the use of antisera as a means of antigen identification.

It was my intention, subsequent to antigen identification, to purify the antigen(s) to homogeneity and carry out an initial screening of a cDNA expression library. For several reasons, this has unfortunately not been possible. Although not reported here, a cDNA expression library to *Rh. appendiculatus* unfed females has been set up, but as yet has not been screened.

2 Materials and Methods.

2.1 Ticks.

Rhipicephalus appendiculatus ticks (fig. 2.1) used in this work were from a laboratory colony maintained at the School since August 1977. The colony was established from engorged pathogen-free females obtained from Kabete, Kenya.

2.1.1 Maintenance of Non-Parasitic Stages.

Unfed larvae as well as engorged females which had detached from animals were kept in glass specimen tubes, 3.5cm x 2cm (I.D.) closed with 4.5cm² pieces of fine meshed nylon gauze and stoppered with polythene caps with a 0.75cm diameter aeration hole. Fed larvae/unfed nymphs were maintained in glass specimen tubes for stock or kept for preparation of unfed nymphal homogenates in polystyrene cups (250ml volume), covered with 15cm² pieces of fine meshed nylon gauze, and fitted with lids each with a 3cm aeration hole in the middle.

Tubes and cups containing ticks were kept in large perspex dessicator cabinets (Gallenkamp). A relative humidity of 80% was maintained in the desiccator by placing a reservoir of 20%(w/v) potassium hydroxide in distilled water in the lower part of the desiccator. Desiccators were located on a bench surrounded by a Risella oil moat in a room, and maintained at a constant

Figure 2.1 *Rhipicephalus appendiculatus* Adults;
Male and Female (x 10).



temperature of 25°C ($\pm 1^\circ$), and a constant 12hr photoperiod provided by two 80 watt and one 40 watt fluorescent tubes connected to a Venner daily time switch.

2.1.2 Maintenance of Parasitic Stages

Outbred female albino guinea pigs (Dunkin Hartley strain) and female New Zealand white rabbits were obtained from commercial suppliers and used for feeding ticks. The guinea pigs ranged in weight from 370 - 600g and the rabbits from 2.0 to 3.5kg, and during the feeding period were kept in rooms maintained at 25°C ($\pm 1^\circ$) and 12hr photoperiod. They were given food pellets (Quest Nutrition) and water *ad libitum*. Guinea pigs were used in immunisation and tick challenge experiments and also for stock feeding of ticks (to maintain the colony). Rabbits were used principally for stock feeding. Unless otherwise stated, each animal was used only once and painlessly killed after completion of tick feeding by injection of sodium pentobarbitone BP, 200mg in 1ml (veterinary "Euthatal", May and Baker).

Guinea pigs and rabbits were prepared for feeding by anaesthetising by intramuscular injection into the dorsum of upper hind leg with alphaxalone/alphadolone acetate ("Saffan" Glaxo) at the dose of 1ml.kg^{-1} body weight. Using an electric hair clipper (Oster) a dorsal area of ca. 25cm^2 in guinea pigs and 50cm^2 in rabbits was shaved, to expose the skin, which was then disinfected with ethyl alcohol to kill any surface bacteria

likely to cause secondary infection of the tick feeding lesions. Cylindrical feeding bags made of heavy duty drill (length 12cm x diameter 11cm for guinea pigs; 16cm x 14cm for rabbits) were attached to the shaved feeding area with a water based latex adhesive ("Tip-on", Pertec Ltd.) and secured to the body with zinc oxide adhesive tape. Rigid polythene (rabbits) or corrugated cardboard (guinea pigs) collars were fastened round the necks of the animals to prevent chewing or scratching of the feeding sleeves, and the animals kept for 24hr to allow full recovery from the anaesthetic and for the glue to dry.

2.1.2.1 Stock Feeding of *Rh. appendiculatus*.

Upto 1000 unfed larvae, 500 unfed nymphs or 15 unfed males and 10 unfed females were released on each guinea pig and upto 7500 unfed larvae, 5000 unfed nymphs or 25 unfed males and 20 unfed females on each rabbit. In the case of larvae and nymphs the tubes were placed unopened inside the feeding bag, the bag sealed tightly with zinc oxide tape and the lid(s) removed from the tube(s) by manipulating them from the outside. Adult ticks were tapped down directly from the tube into the feeding bag. In the case of adult ticks, the bags were opened daily from day 5 after release, and any detached engorged females removed and placed in groups of up to 10 in vented plastic petri dishes (9cm), and placed in a desiccator for oviposition. After all the females had detached, the males were carefully removed and killed in ethyl alcohol. Fed larvae were collected in bulk daily from the

third day after release, and transferred when convenient into tubes during the quiescent nymphal premoult or left in cups to moult. Fed nymphs were collected in bulk from day 3 and upto 300 placed in each plastic cup, to moult into adults. The required number of adults were transferred to glass tubes for release on animals.

2.1.2.2 Tick Challenge Experiments on Immunised and Non-Immunised Animals.

Fifteen unfed *Rh. appendiculatus* males and 10 unfed *Rh. appendiculatus* females (usually 1 - 2 months old and from the same batch for all animals in a particular experiment) were released on each prepared guinea pig, the sleeves were opened daily from the sixth day onwards and any detached females collected and weighed individually using an electronic balance (Oertling R52). All references in this thesis to engorged tick weight are for engorged female weight. They were then separately tubed and placed in a desiccator. Once all the females had detached, the males were removed and killed in ethyl alcohol. The onset of egg laying, the weight of the egg mass and the egg hatch rate were recorded for each female. One week to 10 days after the last female had dropped off, guinea ^{pigs} were bled by cardiac puncture and killed painlessly. Sera were separated as in 2.5 and stored at -20°C . In one experiment, both immunised and non-immunised animals were kept for a second tick challenge feed.

2.2 Preparation of Tick Extracts.

2.2.1 Unfed *Rh. appendiculatus* Larvae, Nymphs or Adults and Partially Fed or Fully Fed Females.

Two week old unfed larvae, two week old unfed nymphs or two or three week old unfed adults were killed by chilling in liquid nitrogen for 1 minute. Partially fed females (6 days) which had been forcibly removed from hosts and fully engorged females which had detached from their host were killed by chilling in liquid nitrogen. Egg shells or cuticles were cleaned from the killed ticks which were poured into a porcelain mortar (capacity 600ml, diameter 15cm) chilled with liquid nitrogen. Liquid nitrogen was poured onto the ticks and these were ground with a prechilled porcelain pestle. Fresh liquid nitrogen was added twice more, the material being ground after each addition, and the grindate was transferred to a fresh mortar (200ml, 10cm) (chilled on ice) with the aid of a polypropylene spatula. From this point all procedures were carried out at 4°C. A small volume of sterile PBS (phosphate buffered saline) pH 7.3 (Appendix 1.A) containing 1mM EDTA (disodium salt) (Sigma) was added and the material homogenised for a further five minutes. Additional PBS-EDTA was added and the resultant homogenate transferred to a chilled polypropylene centrifuge tube (Beckman). The mortar, pestle and spatula were all carefully washed several times with PBS-EDTA and the washings added to the centrifuge tube. Enough PBS/EDTA was added to give a *ca.* 10% w/v homogenate. The tube was packed in an ice-bath and the homogenate sonicated three times at 18µm in

ten second bursts separated by intervals of 60 seconds (MSE Mark 2 Ultrasonic Disintegrator), after which the homogenate was centrifuged at 27 000g for one hour at +4°C (Beckman J2-21 centrifuge + JA-20 rotor). The supernate (extract) was carefully decanted into a chilled sterile polystyrene universal container (Sterilin), and the pellet discarded. The extract was filter sterilised by passing it sequentially through 0.45 and 0.22µm low protein binding durapore membranes (Millex HV non sterile and Millex GV sterile filter units, Millipore), aliquoted into sterile cryotubes (Nunc) and frozen at -70°C.

In some of the earlier preparations the ticks were killed by chilling at -70°C for 30min, and then homogenised in a small volume of PBS (no EDTA) in a mortar chilled on ice. All other procedures were as described above.

2.2.2 Unfed Larvae of *Am. hebraeum*, *Am. variegatum*, *Bo. microplus* and *Rh. e. evertsi*.

Larvae were killed by chilling at -70°C for 30min and homogenised in a small volume of PBS in a mortar chilled on ice. All other procedures were as described in 2.2.1.

Am. hebraeum and *Am. variegatum* larvae were from colonies maintained at the School since 1976 and 1977 respectively, and originating from Gulu Farm, East London, South Africa (*Am. hebraeum*), and Kabete, Kenya (*Am. variegatum*). *Bo. microplus*

larvae were supplied by Coopers Animal Health, Berkamsted, Herts. *Rh. e. evertsi* larvae were from a colony maintained at the School since 1986, and originally from a laboratory colony maintained at the Medical University of Southern Africa, South Africa.

2.2.3 *Rh. appendiculatus* Salivary Gland Extract.

Rh. appendiculatus unfed males and females were released on the backs of rabbits as in 2.1.2, and 6 days later, the partially fed females were forcibly removed, measured and weighed, and briefly washed in PBS pH 7.3. The females were dissected under cold PBS using a Weck microscalpel with a cutting angle of 45°. The upper cuticle was carefully removed and the salivary glands transferred to a prechilled well dish containing a small volume of PBS. Once all the females had been dissected the glands were cleaned of other tissues, and the contents of the well dish were homogenised in a small volume of PBS (0.1ml to 1 pair of glands) in a Jencons 5ml glass tissue grinder before being spun in a polypropylene centrifuge tube at 1200g for 30min at +4°C (Beckman J2-21, JA-20 rotor). The supernate was removed, filtered through a Millex GV 0.22µm sterile filter unit, aliquoted into sterile cryotubes and frozen at -70°C.

2.2.4 *Rh. appendiculatus* Gut Extract.

The method used was identical to that in 2.2.3, except that gut caecae were used instead of salivary glands.

2.3 Purification of Whole Unfed Larval or Whole Unfed Nymphal Extracts.

2.3.1 Charcoal Treatment.

Activated charcoal readily absorbs fats and lipids. It was hoped that in addition to removing any lipid content from the extract, it may have removed some or all of any lipoproteins present.

The required quantity of prepared extract was removed from storage at -70°C , rapidly thawed, mixed by gentle swirling and spun in a polypropylene centrifuge tube at $1000g$ for 20min at $+4^{\circ}\text{C}$ (Beckman J2-21 centrifuge, JA-20 rotor). Activated charcoal (Sigma) was added to the extract at the rate of 1.5% w/v and the extract gently agitated, using an end-over-end stirrer (Luckhams), for 10min after which the charcoal was removed by vacuum filtration through a glass sinter (Gallenkamp, No. 1), centrifuged at $1000g$ as before and the extract passed through a $0.22\mu\text{m}$ durapore membrane as in 2.2. One extract of unfed larvae was treated in this manner.

2.3.2 Removal of Low Molecular Weight Proteins by Dialysis.

Extensive dialysis of extract through a semipermeable membrane permits removal of any material that is smaller than the pore size of the membrane.

Visking dialysis tubing (Medicell) with a molecular weight exclusion limit of 12 - 15kDa was prepared by soaking overnight at +4°C in distilled water containing 0.02% sodium azide. The tick extracts were thawed and centrifuged as in 2.3.1 and transferred into the dialysis tubing (sealed at one end with a Pierce dialysis clip) with the aid of a sterile pastette (Alpha). The top end of the tubing was sealed with a dialysis clip, and the extract extensively dialysed against PBS pH 7.3 (10ml extract : 2l PBS 3x) at +4°C. After dialysis the extract was transferred to a polypropylene centrifuge tube, spun and filtered as in 2.3.1. One extract of unfed larvae had low molecular weight proteins removed by dialysis.

2.3.3 Precipitation with Saturated Solutions of Ammonium Sulphate.

"Salting-out" of proteins, using high concentrations of salts, is one of the most widely used techniques in protein purification (Scopes, 1982); ammonium sulphate is the salt most commonly used for this purpose.

The extracts were thawed and centrifuged as in 2.3.1. The protein concentration was adjusted to 5 mg.ml^{-1} by addition of sterile PBS pH 7.3 (Appendix 1.A) and the sample placed in a sterile glass universal container. With continuous gentle stirring an equal volume of saturated ammonium sulphate solution (Appendix 1.C) was added dropwise, one drop every 10 secs. Stirring was continued for a further 10min after which the sample was left to flocculate for 6hr; it was then spun at $6000g$ for 20min. The supernate was carefully removed and the pellet (precipitate) was resuspended in a small volume of sterile PBS. Both supernate and precipitate were dialysed 3 times, first against deionised water and then twice against PBS. The supernate was concentrated against crystalline PEG 8000 (Sigma) and then both supernate and precipitate were centrifuged as in 2.3.1 and passed sequentially through Millex AA $0.8\mu\text{m}$ and Millex GV $0.22\mu\text{m}$ sterile filter units. All procedures were at $+4^{\circ}\text{C}$. One extract of unfed larvae was fractionated with saturated ammonium sulphate solution.

2.3.4 Precipitation with Polyethylene Glycol.

In addition to salts (and organic solvents) aggregation of proteins without denaturation can be achieved using organic polymers such as polyethylene glycol (Scopes, 1982).

The extract was thawed and centrifuged as in 2.3.1. After adjusting the protein concentration of the extract to 5mg.ml^{-1} , a solution of 20% polyethylene glycol 8000 (Sigma) was

added in a ratio of one part PEG : four parts extract (v/v). After thorough, but gentle, mixing the material was left to precipitate at +4°C overnight, following which it was centrifuged as in 2.3.3 and the pellet discarded. The supernate was dialysed extensively against PBS before filtering as in 2.3.3. One extract of unfed larvae was purified with PEG 8000.

2.3.5 Precipitation with Solid Ammonium Sulphate.

The use of saturated solutions of ammonium sulphate (2.3.3) is impractical when large volumes are involved and if levels of saturation above 50% are required. By adding crystalline ammonium sulphate directly into the protein solution saturation levels of upto 100% are attainable without any large increases in volume. This method has the additional advantage that it is possible, by step-wise addition of ammonium sulphate, to partition the extract into several fractions.

The required quantity of prepared extract was removed from storage, thawed and spun as in 2.3.1. The protein concentration of the sample was adjusted to 10 mg.ml⁻¹ with sterile PBS pH 7.3 and the sample placed in a sterile glass serum bottle (Gibco). With continuous gentle stirring crystalline ammonium sulphate (Fisons, specially low in heavy metals) was added slowly over a 10 minute period to give the required ammonium sulphate concentration (w/v, see Appendix 1.D). Stirring was continued

for 10min more and the sample left to flocculate for 30min; it was then spun at 6000g for 20min. The supernate was carefully removed and either dialysed, concentrated, spun and filtered as in section 2.3.3, or placed in a fresh serum bottle and additional ammonium sulphate added to further fractionate the extract. The precipitate(s) from each step were resuspended and processed as in section 2.3.3. In this manner extracts were partitioned into 4 fractions based on the solubility of the proteins. All procedures took place at +4°C. Three extracts of unfed nymphs were fractionated by addition of ammonium sulphate to w/v concentrations of 20%, 35% and 45% (corresponding to 34%, 55% and 67.5% saturation respectively).

2.3.6 Size-Exclusion High Performance Liquid Chromatography (SEHPLC).

This method of protein separation relies upon the molecular sieving effect of a gel cast in bead form, and having a three-dimensional open, cross-linked molecular network. Smaller molecules are able to penetrate the pores in the beads whilst larger molecules are not; subsequently smaller molecules are delayed in their passage through a column of such beads whilst larger molecules can pass quickly through (Scopes, 1982).

The extract was thawed and centrifuged as in 2.3.1, the protein concentration adjusted to ca. 7mg.ml⁻¹ and the sample filtered through a 0.22µm durapore membrane. One ml of extract was loaded

via a 1ml sample injection loop onto a 300mm x 7.5mm TSK G3000SW size-exclusion column (Toya Soda) fitted with a TSK SW guard column connected to a Beckman HPLC system. Protein coming off the column was detected by measuring absorbance of the column eluate at 280nm with a Beckman UV monitor connected to a Spectra Physics SP 4270 integrator. The system was operated at room temperature, the sample eluent was HPLC-PBS (Appendix 1.B) and the flow rate through the column $0.8\text{ml}\cdot\text{min}^{-1}$. The eluate was collected in sterile polystyrene universal containers (Sterilin), kept on ice, in fractions corresponding to the 3 principal peaks: high (ca.700 to 130kDa), medium (130 to 20kDa) and low (<20kDa) molecular weight. Several runs were made, and the fractions corresponding to identical peaks pooled. Fractions 1 (high) and 2 (medium) were dialysed against 10% PBS at $+4^{\circ}\text{C}$ and then all 3 fractions were concentrated with PEG 8000 before filtering through a $0.22\mu\text{m}$ durapore membrane. Molecular weights were estimated by determining the elution times of protein standards (Bio-Rad). Three extracts of unfed nymphs were fractionated by SEHPLC. Fractions were stored at -70°C until use.

Analytical SEHPLC was carried out under conditions identical to above, except that $20\mu\text{l}$ of sample was loaded via a $20\mu\text{l}$ sample injection loop onto the column.

2.3.7 Precipitation of Immune Complexes.

When antigen and antibody are mixed in the correct proportions an antigen-antibody complex is precipitated (Roitt, 1988).

Four ml of unfed nymphal extract was thawed and centrifuged as in 2.3.1 and 2ml placed in each of 2 sterile polystyrene bijoux (5ml, Sterilin). Into one bijou was added 2ml of immune rabbit serum (2 infestations with *Rh. appendiculatus* larvae) and into the other 2ml of immune guinea pig serum (2 sequential infestations with *Rh. appendiculatus* larvae) and the contents of the individual bijoux mixed on a whirlimixer at 5min intervals for 30min. The mixtures were each made up to 5.5ml with PBS pH 7.3, and each transferred into 2 Beckman ultraclear centrifuge tubes and spun in a Beckman L8-80 ultracentrifuge (SW.55 rotor) at 70 000*g* for 1hr at +4°C. The supernates were carefully removed, the pellets washed in cold PBS and each resuspended in 2.5ml cold PBS. The complexes were stored at -70°C until use.

2.3.8 Ultracentrifugal Fractionation of Membrane Bound and Soluble Proteins.

It is possible to pellet proteins that are membrane associated as they are in an aggregated form.

An extract of unfed nymphs was prepared as in 2.2.1 except that after centrifugation at 27 000*g*, 10ml of the supernate was transferred, unfiltered, into 2 Beckman ultraclear centrifuge

tubes and spun at 100 000g at +4°C in a Beckman L8-80 ultracentrifuge (SW.55 rotor) for 1hr. After spinning, the supernate was carefully removed and the pellet resuspended in 5% nonidet P40 (Sigma) in PBS pH 7.3 containing 1mM EDTA. Membrane bound proteins were extracted by incubating the resuspended pellet at 37°C for 90min, following which the sample was dialysed and centrifuged as in 2.3.3.

2.4 Immunisation of Guinea Pigs for Challenge Feed by Adult Ticks.

2.4.1 Tick Extracts.

Tick extracts were inoculated at a dose of 1.5mg.kg^{-1} body weight of guinea pig unless otherwise specified (see 3.6). Extracts were mixed with an equal volume of Freund's incomplete adjuvant (Gibco BRL) by passing back and forth 100x through a double-headed needle. Control animals were inoculated with FIA only. Two or 3 animals were immunised, with each extract, by subcutaneous inoculation into the shaved, alcohol sterilised, upper dorsum of the right hind leg. A second similar inoculation was made 14 days later into the upper dorsum of the left hind leg, and a third inoculation, without adjuvant, was given into the right shoulder area at 28 days. Ten to 14 days later, feeding bags were fixed on the backs of the guinea pigs (2.1.2) in preparation for the release of 15 *Rh. appendiculatus* males and 10 *Rh. appendiculatus* females a day later. The ticks were released, collected, weighed etc. as described in 2.1.2.

Subsequent to experiment 3.6.7, the use of FIA was superseded by the use of "Quil A" (Superfos, Denmark), a glycoside obtained from a crude saponin extract of the South American tree *Quillaia saponaria* Molina. This was used at the dose, *per* inoculation, of 50mg (dissolved in 0.1ml PBS pH 7.3) *per* guinea pig, mixed with extract in a bijoux 30min before use. Control animals received 50mg "Quil A" in PBS only.

2.4.2 Nitrocellulose antigen-bearing particles.

(Adapted from the method of Abou-Zeid *et al.*, 1987)

Nitrocellulose is non toxic and proteins bind tightly to it. It is possible to disrupt the integrity of the membrane whilst still conserving any proteins that may be bound to it.

A total of six polyacrylamide gels were prepared, electrophoresed and blotted as in sections 2.7 and 2.8., except that 1.5mg extract were loaded onto each gel. A 0.5cm strip from each of the six blots was stained using the method described in section 2.9 (using serum from immunised guinea pigs), and the region corresponding to 85 - 100kDa was cut-out from each of the NCP sheets using a clean scalpel (because of the high loading of extract on each gel there was considerable overlapping of bands). The strips were gently blotted with filter paper (Whatman 3MM) to

remove excess PBST buffer ("Appendix 1.F"), placed in a porcelain mortar and ground to a powder with a pestle after addition of liquid nitrogen. The NCP was ground twice more under liquid nitrogen to produce a fine powder consisting of nitrocellulose antigen-bearing particles which was split into 3 equal aliquots, 2 of which were stored at -70°C until use. The other aliquot was suspended in 3ml PBS and mixed with 0.5ml FIA using a double-headed needle as in 2.4.1. Three guinea pigs were inoculated as in 2.4.1. The NCP/FIA mixture proved highly viscous and very difficult to inoculate, subsequently for the second and third sets of inoculations the aliquots were suspended in PBS, but no FIA was used.

2.5 Preparation of Antisera.

Blood was collected by cardiac puncture from guinea pigs and rabbits after one, two and three sequential infestations with *Rh. appendiculatus* adults, and also from guinea pigs immunised with crude extracts or their fractions both before and after a challenge feed by adult ticks. In all cases blood was collected into Corvac integrated serum separator tubes, allowed to clot at room temperature for a minimum of 30min and centrifuged at $1200g$ for 30min at room temperature (Beckman TJ-6 bench top centrifuge) to obtain sera, which were collected and stored at -20°C .

2.6 Partial Purification of Serum Immunoglobulins by Ammonium Sulphate Precipitation.

The serum to be precipitated was clarified by centrifugation at 1000g for 20min (as for extracts in 2.3.1) and placed in a sterile bijou. With continuous gentle stirring a volume of saturated ammonium sulphate (Appendix 1.C) equal to that of the serum was added dropwise, one drop every 10 secs. Stirring continued for a further 10min whereupon the sample was left to flocculate for 2hr, after which the sample was spun at 6000g for 20min. The supernate was removed and the pellet was carefully washed with a small volume of saturated ammonium sulphate and respun at 6000g, after which the pellet was resuspended in half the volume of the starting serum. The resuspended precipitate was then extensively dialysed, centrifuged and filtered as in 2.3.2.

2.7 Electrophoretic Separation of Proteins on Polyacrylamide Gels in the Presence of Sodium Dodecyl Sulphate.

(Adapted from the method of Laemmli, 1970)

For buffer and gel recipes see Appendix 1.D. A 2-slab vertical electrophoresis unit (LKB 2001) with a tapwater cooling coil was used. A discontinuous buffer system was utilised, the upper stacking gel was pH 6.8 and the lower separating gel was pH 8.9. The electrophoresis buffer was TRIS glycine and had a pH of 8.3. Both upper and lower gels and the electrophoresis buffer contained 0.1% SDS. For section 3.4, the stacking gel contained

4.5% acrylamide (4.5% T, 0.12% C) and the separating gel contained 5% (top) to 20% (bottom) acrylamide (5% to 20% T, 0.13% to 0.52% C) present as a linear gradient. In all other cases the values were 3.08% acrylamide (3.08T%, 2.6%C) and 12% acrylamide (12%T, 2.6%C) stacking and separating gels respectively. All samples (of variable volume), including molecular weight marker proteins, were prepared in a solvent containing 0.0625mM TRIS-HCl buffer pH 6.8, 2% SDS, 2mM PMSF, 1mM EDTA, 10% glycerol, 0.04% bromophenol blue and 5% 2-mercaptoethanol; and were heated for 2min in a boiling water bath, cooled and carefully loaded onto the stacking gel. Gels were electrophoresed from top (cathode) to bottom (anode) at 160V, max current and power, for 5hr, after which the gels were either fixed and stained as in Appendix 2, or blotted as in 2.8. A molecular weight marker lane was included in every gel.

2.8 Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheets (Western Blotting).

(Adapted from the method of Towbin *et al.*, 1979)

See Appendix 1.F for buffer recipes. Samples were separated by SDS-polyacrylamide electrophoresis, as in 2.7. Upon cessation of electrophoresis, gels were incubated in transfer buffer for 2 x 20min before being sandwiched against nitrocellulose paper (NCP) (Schleicher and Schuell, 0.45 μ m) that had been presoaked (20min) in transfer buffer. The blot sandwiches were placed in a cooled electroblotting tank (LKB 2005, Transphor) filled with

transfer buffer so that the gel was cathodic and the NCP anodic. To maximise efficient transfer of material from gel to NCP, the voltage was set to 10V for 1hr, followed by 30V overnight (ca. 14hr) and finally 100V for 1hr. Gels were then fixed and stained for residual proteins using Coomassie Brilliant Blue R-250 (see Appendix 2) and the blots (NCP) were quenched in PBST buffer (pH 7.6) for 1hr, following which the regions corresponding to the molecular weight marker lanes were excised from the blots and marker proteins visualised with Amido Black (see Appendix 2). Blots were then, either processed to determine antigen-antibody interactions (2.9) or used in lectin-binding studies (2.10).

2.9 Analysis of Antigen-Antibody Binding Reactions using Western Blots.

Unbound sites on quenched NCP were blocked by 2 x 30min incubation with 1% casein in PBST buffer, following which the NCP was cut into strips corresponding to individual lanes in the original polyacrylamide gel. NCP strips were incubated for 90min with the appropriate antiserum (diluted 1:200 in PBST) following which they were thoroughly washed with PBST before incubation with horseradish peroxidase conjugated whole anti Immunoglobulins (Dako, rabbit anti guinea pig, ^{or} goat anti rabbit).

or HRP conjugated whole anti IgG (Sigma, rabbit anti guinea pig) diluted 1:1000 in PBST (1hr). After thorough washing with PBST the strips were incubated in peroxidase substrate

buffer (Appendix 1.G) until the reaction reached completion, where upon the strips were washed thoroughly in PBST, before drying between two sheets of blotting paper. All procedures were carried out at room temperature. Dilutions used were those determined as optimal by serial titration. The degree of staining is related to the amount of binding of the first antibody to the antigen (determined by the number of antibody molecules recognising a particular epitope, and the presence of the same epitope on a given antigen molecule).

2.10 Lectin-Binding Studies using Western Blots.

(Adapted from the method of Frommel and Balber, 1987.)

Unbound sites on quenched NCP were blocked by 3hr incubation in PBST, following which the NCP was cut into strips as in 2.9. NCP strips were incubated for 60min with HRP conjugated lectins. Lectin concentrations were $5\mu\text{g}.\text{ml}^{-1}$ in PBST (1mM MnCl_2 , 1mM CaCl_2 and 1mM ZnCl_2 were included as necessary, see table 3.13). An identical set of strips were exposed to lectins that had each been preincubated with an excess of an appropriate specific sugar (2hr room temperature) to determine any non-specific binding to blotted material. After thorough washing with PBST, the strips were developed in peroxidase substrate solution as in 2.9. All procedures were carried out at room temperature.

2.11 Immunostaining of tick sections.

(Adapted from the method of Manyasi, 1987, by Bechara)

Unfed *Rh. appendiculatus* females were dehydrated in ethyl alcohol and embedded in wax using a Histokinette 2000 embedding machine (Reichert Jung), and 4 - 5µm thick sagittal sections were cut using a Reichert Jung microtome with steel knives. Sections were floated onto a warm water surface in a water bath, mounted on glass slides (Chance propper Ltd., select microslides) and dried. After dewaxing in xylene and dehydrating in ethyl alcohol, endogenous alkaline phosphatase activity was blocked with 20% acetic acid. Sections were placed in TRIS buffered saline (TBS, see Appendix 1.H) and incubated for 90min in a humidity chamber with antibody I* diluted 1:100 with normal goat serum (Nordic) diluted 1:20 in TBS. After thorough washing in TBS, sections were incubated for 90min in antibody II (alkaline phosphatase conjugated goat antiguinea pig IgG, H+L chains, Sigma) diluted 1:20 in normal goat serum/TBS. After washing in TBS, sections were incubated for 15min in alkaline phosphatase substrate solution (Appendix 1.H), extensively washed under running tap water and counterstained for 3min with Carazzi's haematoxylin (BDH). Sections were blued under running tap water, mounted in Apathy's mounting medium (BDH) and examined and photographed using a Reichert Jung 'Polyvar' photomicroscope.

* Antibody I: Immunoglobulins partially purified by ammonium sulphate precipitation (see 2.6) from sera raised against material originating from various unfed nymphal extracts of *Rh.*

appendiculatus (NCP antigen bearing particles, SEHPLC fraction 2, ammonium sulphate 45% supernate, reduced dose 45% supernate), or after 3^o adult feeds or from normal guinea pig serum.

2.12 Estimation of Antibody Titres Using an Enzyme-Linked Immunosorbent Assay (ELISA).

(Adapted from the method of Voller^{et al}, 1979)

Plates were coated with 500ng extract/50µl PBS/well and incubated at +4°C overnight, after which they were washed 3x with wash buffer (Appendix 1.I), 200µl/well. Unbound sites were blocked by incubating with wash buffer for 30min at room temperature, after which the plates were again washed x3. The plates were incubated with sera at relevant dilution (s), 50µl/well, 30min at +37°C and again washed x3, after which they were incubated with HRP conjugated antiserum (rabbit antiguinea pig IgG or rabbit antibovine IgG, Sigma, depending on the sera used) diluted 1:1000 in wash buffer, 50µl/well, 30min at +37°C. After washing 3x in wash buffer the plates were incubated with peroxidase substrate solution (Appendix 1.I), 200µl/well, 45min in the dark at room temperature, after which the reactions were stopped by addition of 25µl 2M sulphuric acid/well. The reaction optical densities were read at 490nm using a Dyntatech MicroELISA Autoreader.

2.13 Estimation of Protein Concentration of Extracts.

The long established method of Lowry *et al.* (1951) was used in all cases, except where the presence of detergents made this impracticable, in which case the Pierce BCA protein estimation kit was used. For summaries of both methods see Appendix 3.

2.14 Parameters used in Assessing Tick Feeding Performance.

Of the various parameters for assessing feeding and reproductive performance of adult ticks as indicators of host immunity (*eg.* number of ticks feeding to repletion, feeding period, engorged weight, weight of egg mass, conversion efficiency index - the ability of females to convert bloodmeal into eggs, hatchability of eggs), the most consistent was engorged female weight. This therefore has been used for assessing immunity in my experiments. Weights of egg masses produced by engorged female ticks, fed on experimental and control animals were also indicators of immunity, and were related to the amount of blood ingested. Conversion efficiency index and egg hatch rate, as parameters, were found to be too inconsistent and as such are not referred to.

2.15 Statistical analyses of engorgement weights, egg-laying and egg hatch rate.

All data were subjected to statistical analysis. Engorged weights of ticks, and their resulting egg masses, from experimental and control animals were tested for significance using the Students's t -test. Using Fischer and Yates' statistical tables (t -distribution) the probability value (P) for the appropriate degrees of freedom was read off. The results were considered significant only when $P < 0.05$. To test for significance between engorged tick weights of different extract fractions and the crude extract, tests for the Analysis of Variance were performed using a statistical package. The results were considered significant only when $P < 0.05$.

In two experiments there were several ticks that engorged but failed to lay eggs. The significance of these observations was examined by employing the Chi squared test for a 2×2 contingency table. The results were considered significant only when $P < 0.05$ when read off Fischer and Yates tables for Chi squared distribution.

3 RESULTS.

3.1 Fractionation/Purification of Tick Extracts.

One extract of unfed larvae and eight extracts of unfed nymphs of *Rh. appendiculatus* were fractionated/purified by a variety of methods.

3.1.1 Unfed Larval Extract After Various Treatments (see 2.3.1 - 2.3.4).

14.75mg soluble proteins were treated with activated charcoal and 11.48mg were recovered after treatment, representing 77.8% of the starting material.

Dialysis of 14.75mg soluble proteins resulted in the recovery of 12.35mg, representing 83.7% of the starting material.

After precipitation with PEG 8000, 13.98mg protein remained in solution out of a total of 20.65mg starting material. This was 67.7% of the original material.

When 26.55mg soluble proteins were exposed to 50% saturation with ammonium sulphate, a total of 19.7mg protein (74.2% of starting material) was recovered; 38% of the starting material being precipitated, and 36.2% remaining in solution.

3.1.2 Unfed Nymphal Extract After Fractionation by SEHPLC.

Three extracts were fractionated in this manner (see 2.3.6).

The protein content of fraction 1 represented 23.3% to 38.4% of the starting material with a mean of 30.6% ($\pm 3.57\%$). Fraction 2 represented 25.6% to 31.7% of the original material (mean 28.9% $\pm 1.45\%$), and fraction 3 ranged from 14.8% to 15.1% (mean 15.0% $\pm 0.07\%$). The total recovery of protein in the three fractions ranged from 69.8% to 79.1% of the starting material (mean 74.4% $\pm 2.19\%$).

3.1.3 Unfed Nymphal Extract Partitioned by Addition of Crystalline Ammonium Sulphate.

Three extracts were fractionated by this method (see 2.3.5).

Addition of ammonium sulphate to give 20% w/v, precipitated 10.3% to 11.9% of the starting material (mean 10.9% $\pm 0.41\%$). Further addition of ammonium sulphate to 35% w/v resulted in another 29.3% to 37.1% of the original starting material being precipitated (mean 33.8% $\pm 1.90\%$), and when this was increased to 45% w/v an additional 11.0% to 18.4% was precipitated (mean 14.3% $\pm 1.77\%$). This left 4.1% to 7.3% of the starting material in solution (final supernate) (mean 5.8% $\pm 0.76\%$). The total recovery in the four fractions ranged from 54.7% to 71.2% of the starting material (mean 64.8% $\pm 4.17\%$).

3.1.4 Membrane Associated Proteins in Unfed Nymphal Extract.

Isolation of membrane bound proteins from an UNE (see 2.3.8) resulted in the recovery of 4.55mg protein (5.2% of total) from a starting total of 88mg. The soluble fraction contained 75.2mg protein (85.4% of total). Total recovery in both fractions was 90.6% of the starting material.

3.1.5 Precipitation of Unfed Nymphal Extract Proteins by Complexation with Antibody.

Preparation of immune complexes (see section 2.3.7) resulted in the recovery of <200µg protein for each complex (rabbit and guinea pig). 15.4mg of UNE protein was used for each reaction with antibody, so each complex must contain <1% of UNE proteins complexed with antibody.

3.2 Protein Content of Crude Tick Extracts.

The Lowry method (Appendix 3.A) was used in all cases except for estimation of protein in membrane bound (detergent solubilised) protein, where the Pierce BCA method (Appendix 3.B) was used. Estimates of protein concentration of an UNE by the two methods yielded little difference (8.7mg.ml^{-1} by the method of Lowry, compared to 8.8mg.ml^{-1} using the Pierce BCA method; a difference of <2%).

TABLE 3.1 PROTEIN CONTENT OF *RH. APPENDICULATUS*
EXTRACTS FRACTIONATED/PURIFIED AS IN
SECTION 3.1.

TYPE OF EXTRACT	PROTEIN CONCENTRATION (mg.ml ⁻¹)	FRACTIONATION /PURIFICATION METHOD	REFER TO TABLE
ULE	5.9	various	3.17
UNE	9.65	SEHPLC	3.18
"	10.5	"	3.19
"	10.6	"	3.20
UNE	10.2	ammonium	3.21
"	8.6	sulphate	3.22
"	10.0	fractionation	3.23
UNE	7.7	immune complex formation	3.25
UNE	8.7 (Lowry) 8.8 (Pierce)	membrane protein isolation	3.27

ULE Unfed Larval Extract UNE Unfed Nymphal
Extract SEHPLC Size Exclusion HPLC

TABLE 3.2 PROTEIN CONTENT OF TICK EXTRACTS NOT USED FOR FRACTIONATION/PURIFICATION.

SOURCE	TYPE OF EXTRACT	PROTEIN CONCENTRATION (mg.ml ⁻¹)	REFER TO SECTION
<i>An. hebraeus</i>	ULE	9.7	3.4 3.5.6
<i>An. variegatus</i>	ULE	10.6	3.4 3.5.6
<i>Bo. microplus</i>	ULE	7.5	3.4 3.5.6
<i>Rh. appendiculatus</i>	ULE	8.6	3.4 3.5.1 3.5.6
	UNE	10.0	3.5.1
	"	10.5	3.4 3.5.1
	UAdE	6.35	3.4 3.5.1
	PFFE	12.75	3.4 3.5.1
	FFFE	73.0	3.4 3.5.1
	SGE	0.85	3.4 3.5.1
	GCE	10.6	3.4 3.5.1

ULE Unfed Larval Extract UNE Unfed Nymphal Extract
 UAdE Unfed Adult Extract PFFE Partially Fed Female
 Extract FFFE Fully Fed Female Extract SGE Salivary
 Gland Extract GCE Gut Caecae Extract

Starting protein concentrations (mg.ml^{-1}) of extracts fractionated/purified as in 3.1 are listed in table 3.1. Other tick extracts utilised in my work, and their protein contents, are listed in table 3.2.

3.3 Size-Exclusion HPLC Elution Profiles of Whole Tick Extracts and Their Fractions.

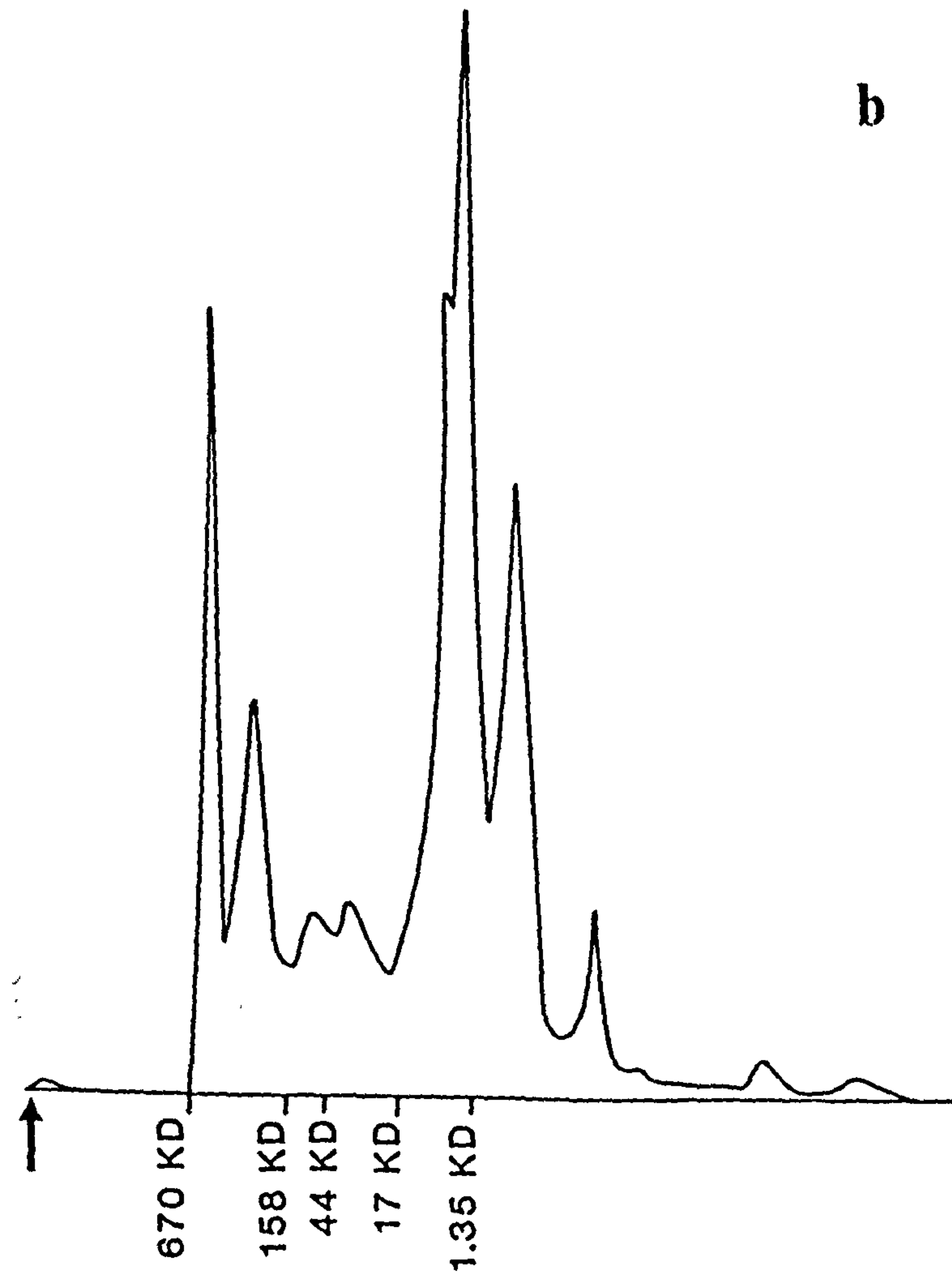
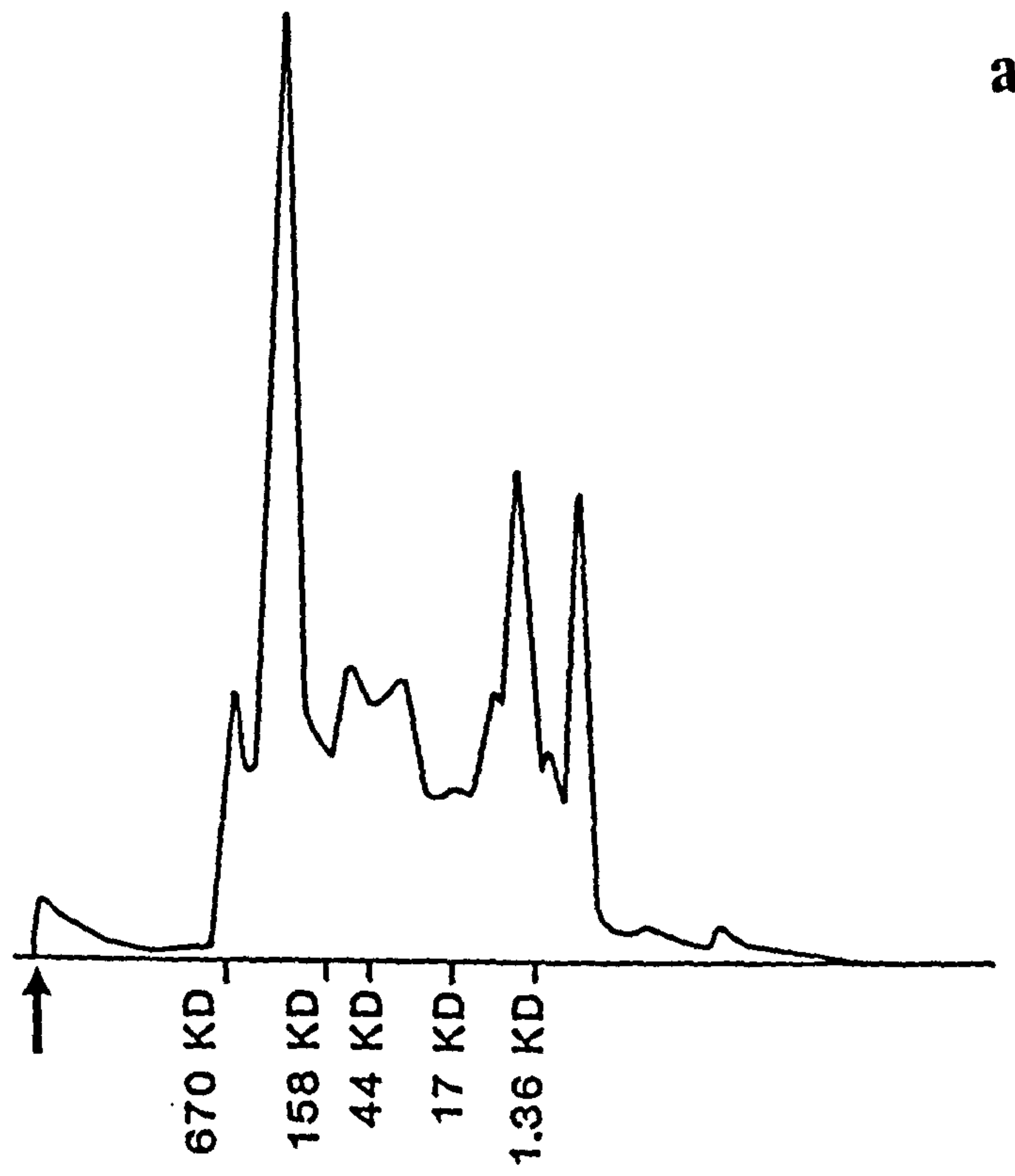
3.3.1 Whole Tick Extracts of Different Species.

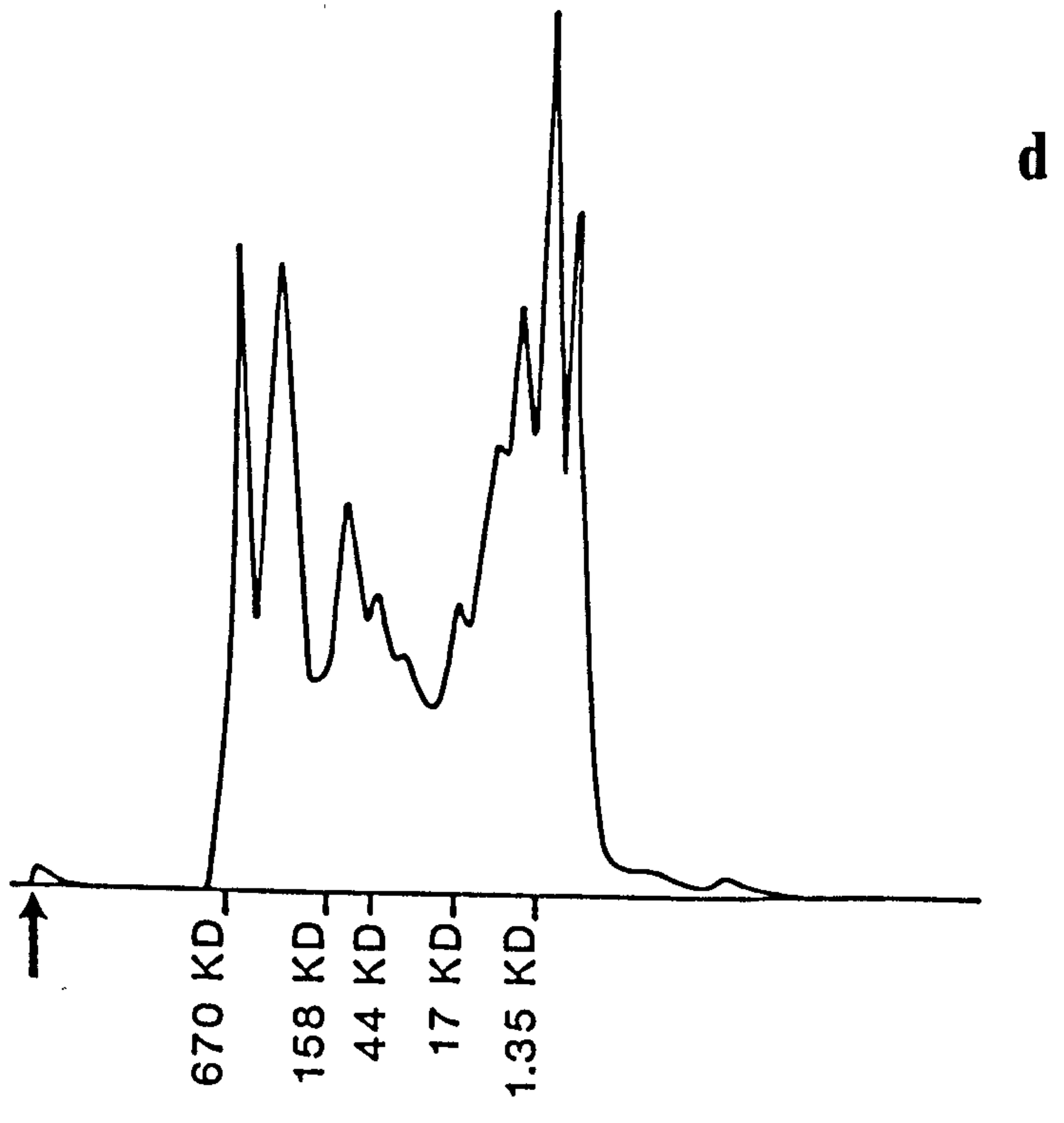
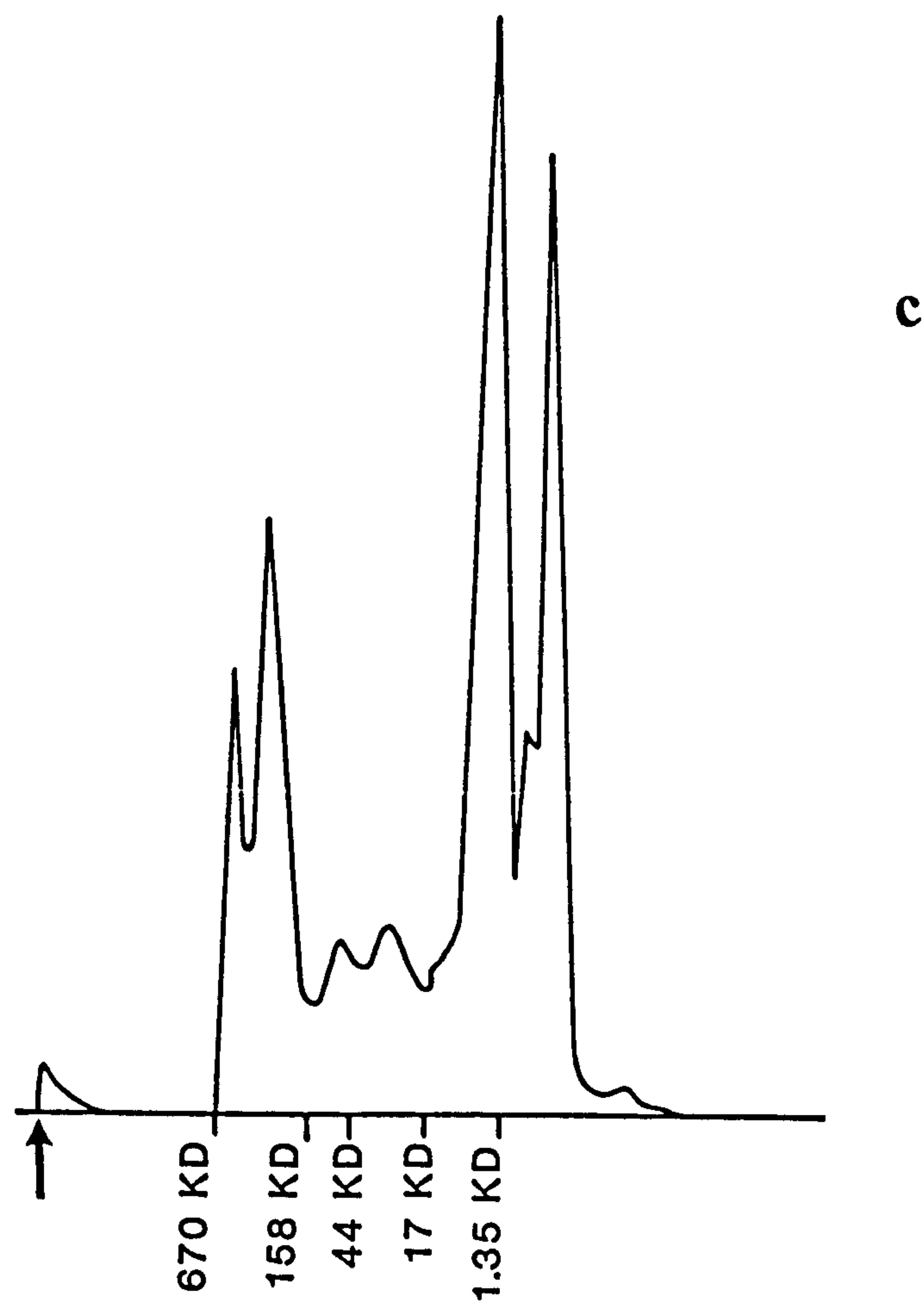
Protein elution profiles of whole extracts of unfed larvae, nymphs and adults of *Rh. appendiculatus* and of unfed larvae of *Am. variegatum* and *Am. hebraeum* are shown in figures 3.3.1a to 3.3.1e. The majority of proteins in all three species were in the high (670 - 158kDa) and low (below 17kDa) molecular weight regions. Proteins of intermediate molecular weight ranged from ca. 17 to 158kDa. Larval extracts of both *Amblyomma* species had protein materials of low molecular weight which were absent in the *Rh. appendiculatus* extracts.

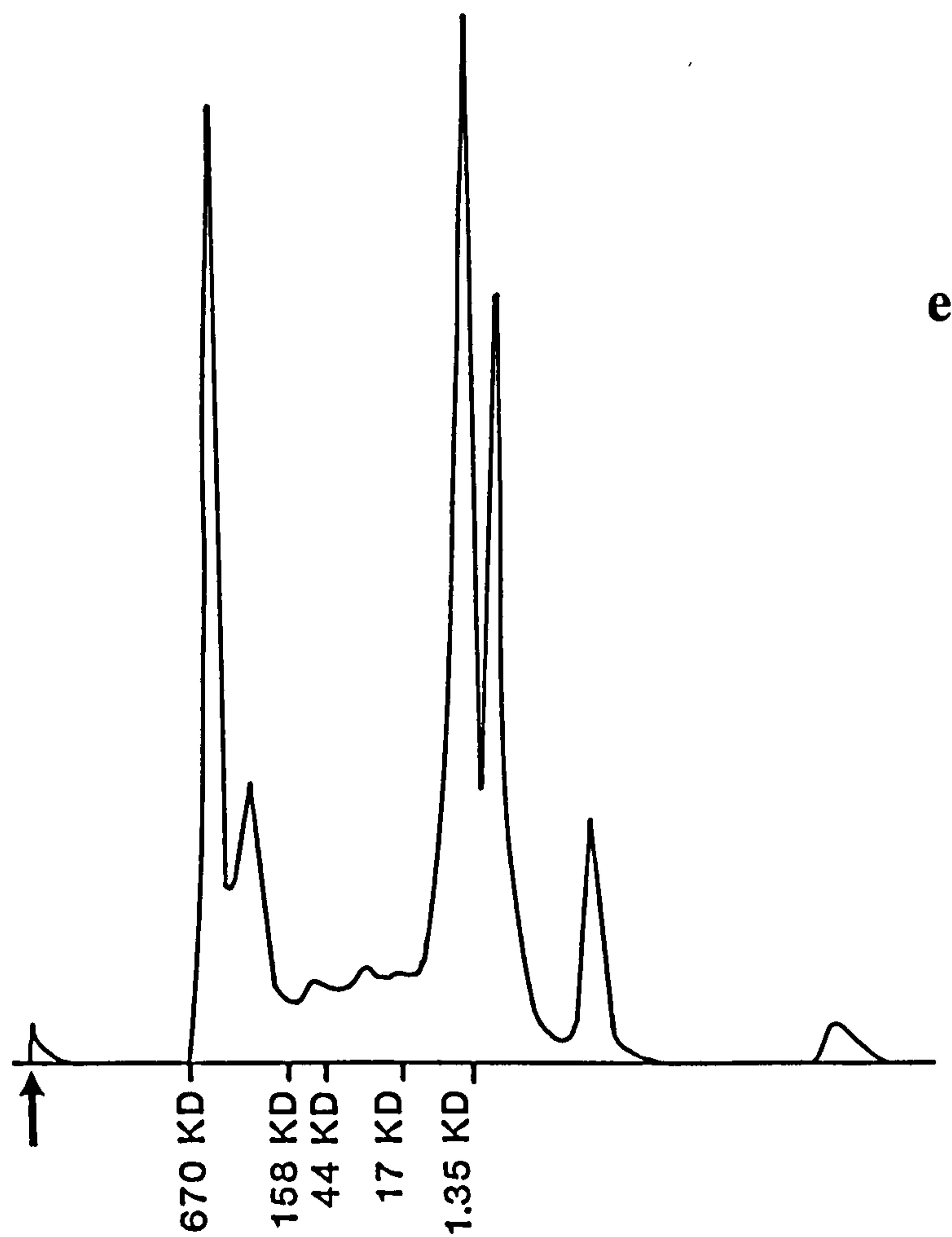
3.3.2 *Rh. appendiculatus* Whole Tick Extracts and Salivary Gland Extracts.

When elution profiles of whole ULE, UNE and UAdE of *Rh. appendiculatus* were compared with that of an extract of salivary glands (SGE) from partially engorged females of the same species,

3.3.1







Figures 3.3.1a to 3.3.1e: Size exclusion elution profiles of whole tick extracts of different species.

- (a) *Rh. appendiculatus* unfed larval extract.
- (b) *Rh. appendiculatus* unfed nymphal extract.
- (c) *Rh. appendiculatus* unfed adult extract.
- (d) *Am. variegatum* unfed larval extract.
- (e) *Am. hebraeum* unfed larval extract.

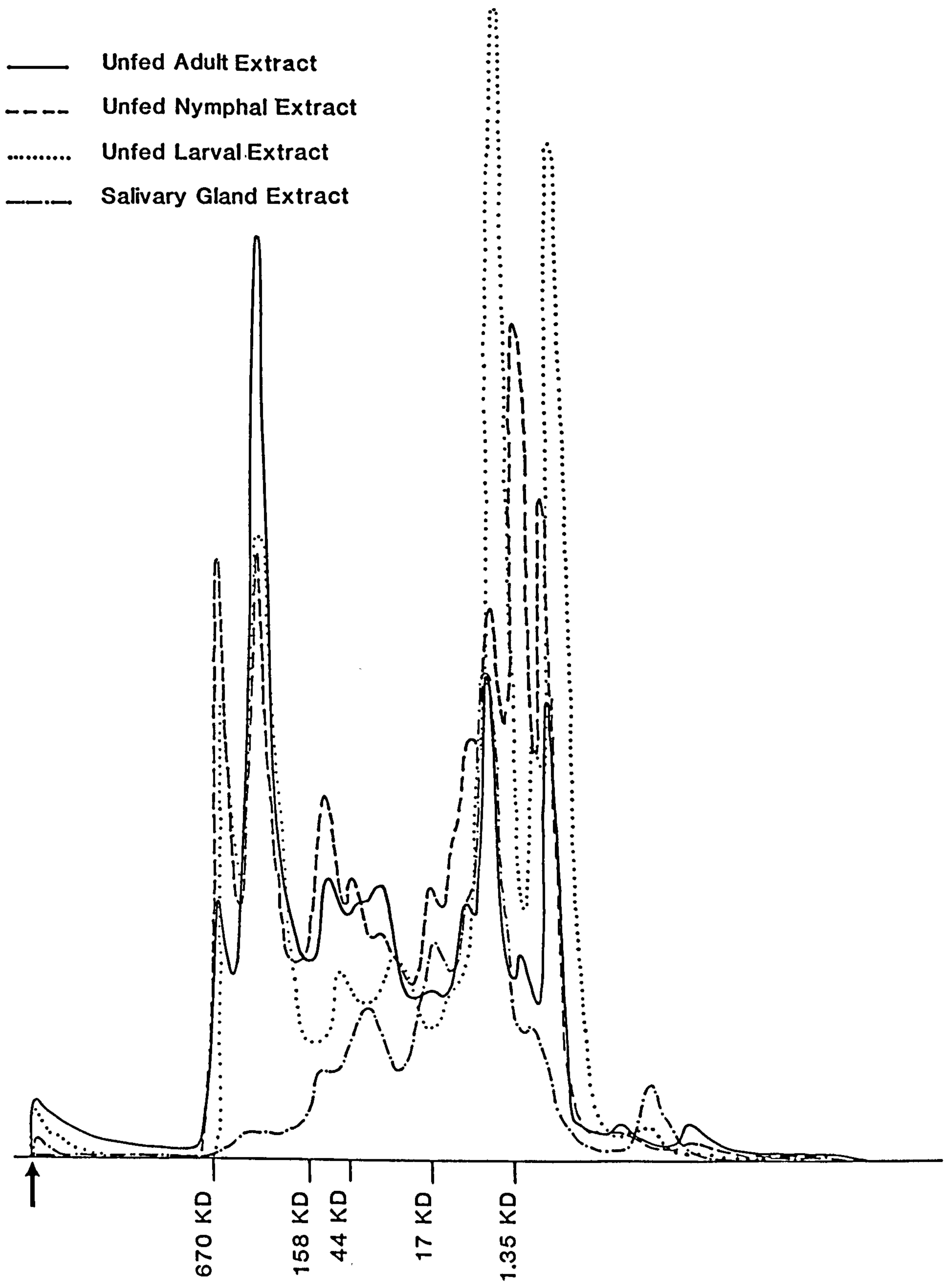


Figure 3.3.2: Size-exclusion HPLC elution profiles of *Rh. appendiculatus* whole tick extracts and salivary gland extract.

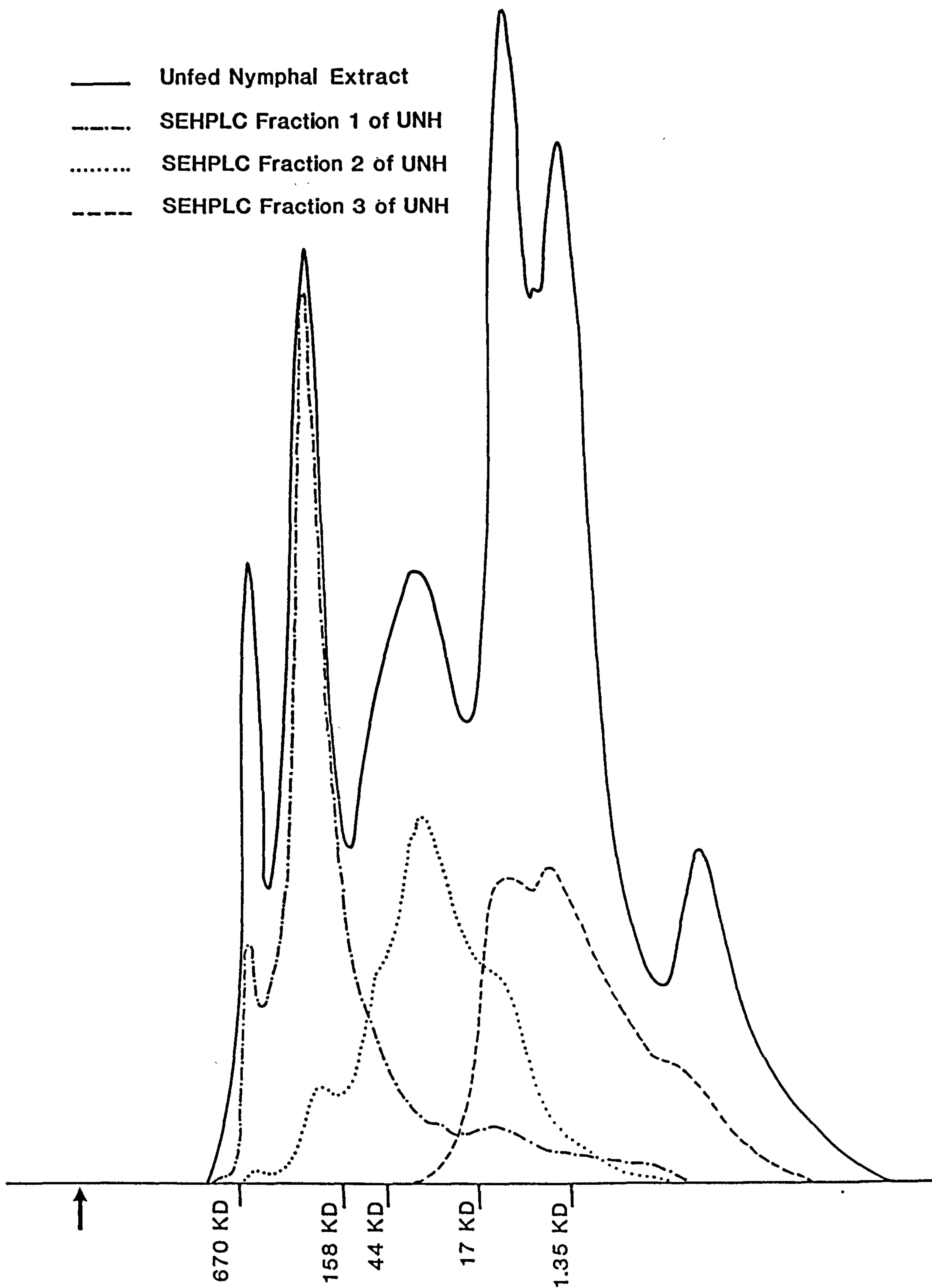


Figure 3.3.3: Size exclusion elution profiles of whole unfed nymphal extract and its SEHPLC fractions.

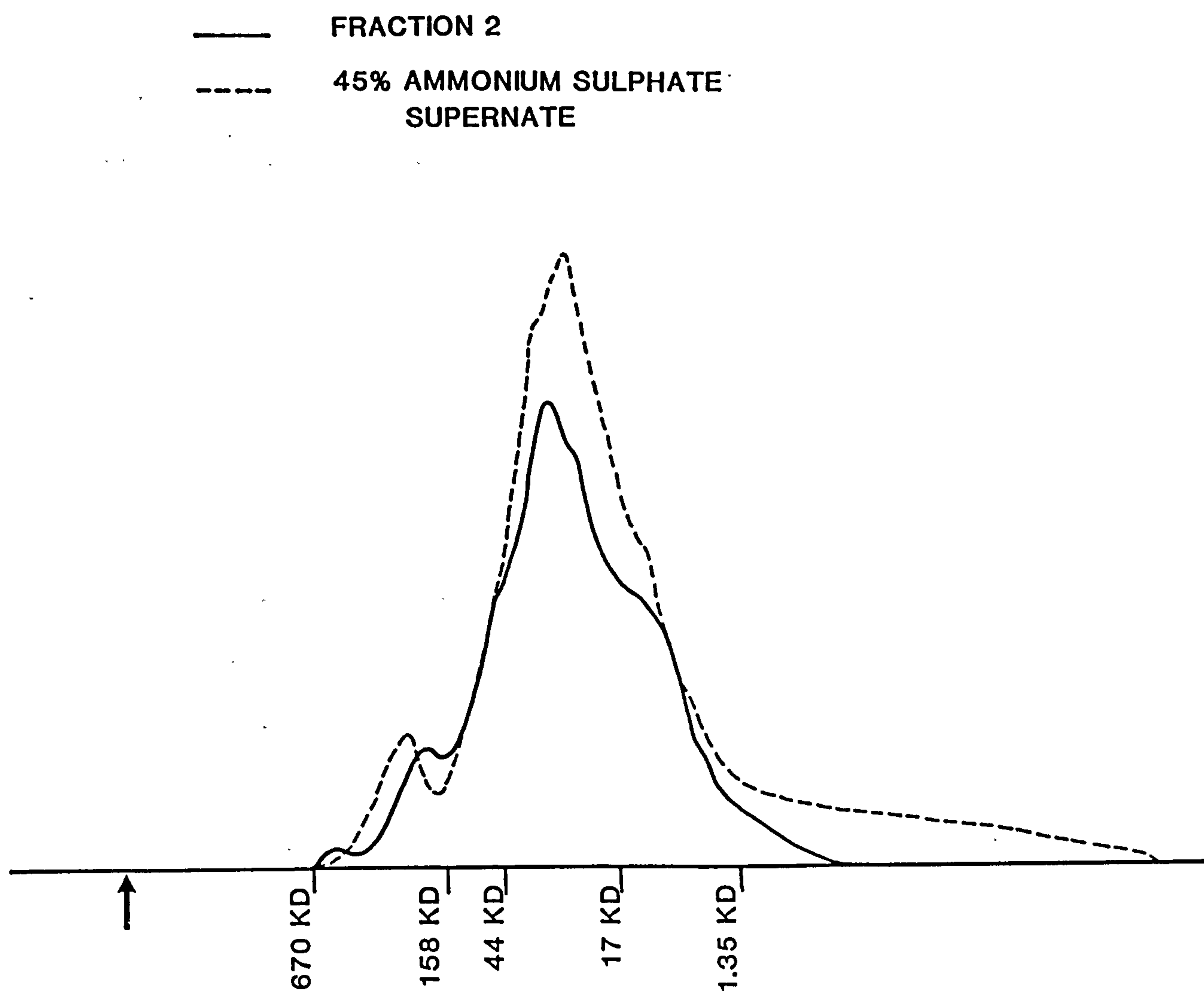


Figure 3.3.4: Size exclusion elution profiles of SEHPLC fraction 2 and the ammonium sulphate 45% supernate fraction of unfed nymphal extract.

it was found that while whole tick extracts were generally more or less similar in their profiles with the majority of proteins in the high and low molecular weight regions, in the SGE there were relatively few proteins above *ca.* 44kDa. None of the high molecular weight peaks were seen in the SGE (figure 3.3.2).

3.3.3 *Rh. appendiculatus* Whole Unfed Nymphal Extract and Its SEHPLC Fractions.

Protein elution profiles of the three fractions of whole UNE obtained by size exclusion HPLC are compared in figure 3.3.3. Although separation was not absolute and there was significant overlap, fraction 1 was composed principally of high molecular weight species (>158kDa), fraction 2 of mainly intermediate molecular weight proteins (158 - 17kDa) and fraction 3 of low molecular weight species (below 17kDa).

3.3.4 *Rh. appendiculatus* UNE SEHPLC Fraction 2 Profile Compared to That of *Rh. appendiculatus* UNE Ammonium Sulphate 45% Supernate.

The elution profiles of the ammonium sulphate 45% supernate fraction and SEHPLC fraction 2, both obtained from the same whole UNE, were found to be very similar (figure 3.3.4). As will be shown later (tables 3.21 and 3.19), both of these UNE fractions gave significantly ($P < 0.05$) higher protection than all other fractions upon immunisation and challenge feeds.

3.4 Electrophoretic Separation of Proteins/Polypeptides in Crude Extracts and Their Fractions.

Proteins were separated on a linear 5 - 20% gradient polyacrylamide gel as described in 2.7 (figure 3.4). In excess of 20 protein/polypeptide bands were observed in crude extracts of unfed *Rh. appendiculatus* and in extracts of partially fed females of *Rh. appendiculatus*. In extracts of fully fed *Rh. appendiculatus* females a minimum of 15 sharp bands could be seen. In all of the extracts the majority of bands were located between 100kDa and 14kDa, relatively few bands were seen above 100kDa. In each case other bands were present, but resolution was such that any estimate of the total number of bands was not possible.

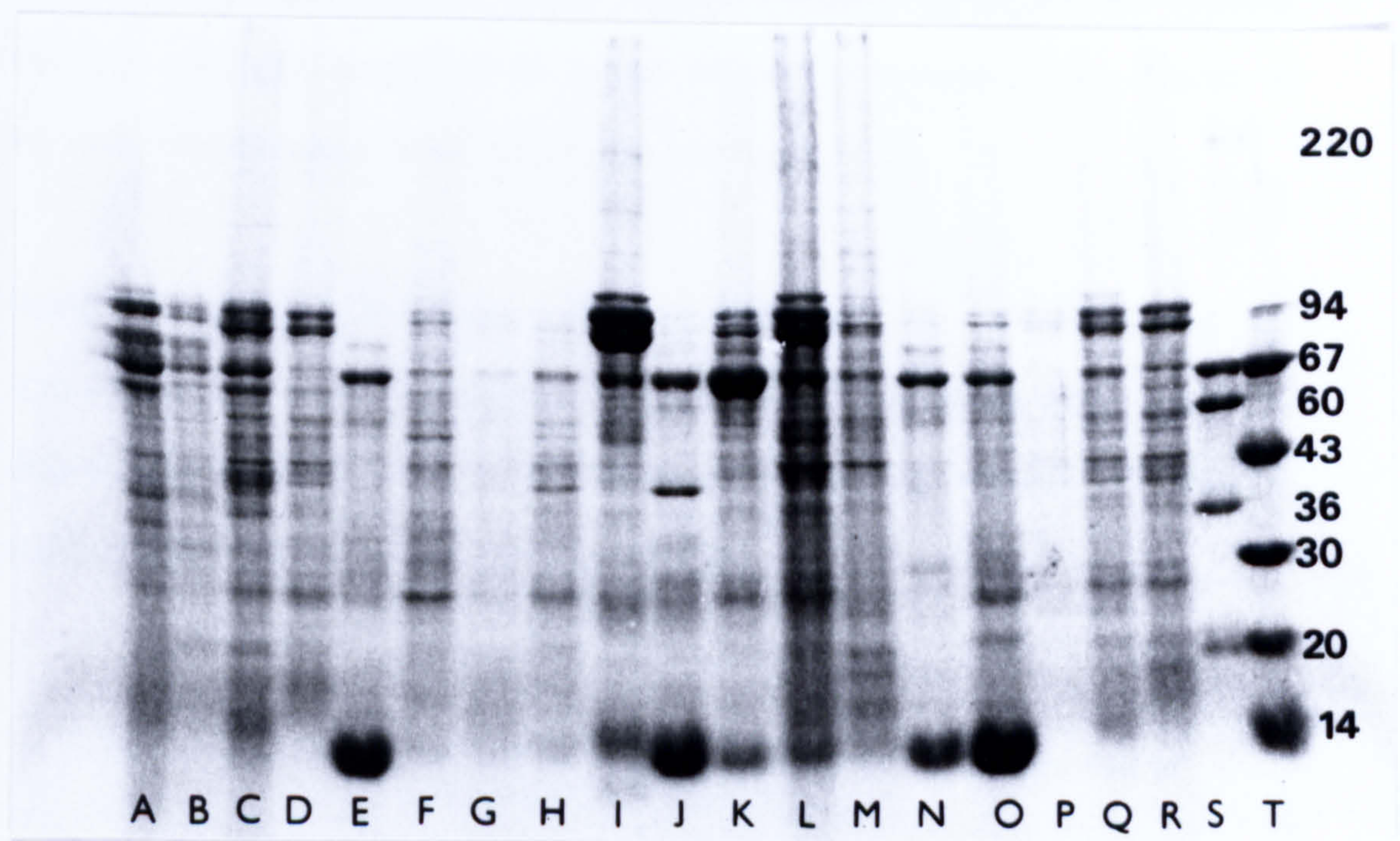
When SEHPLC fractions, used for immunisation (see table 3.19) were separated on SDS-polyacrylamide gels, fraction 1 was observed to have bands mostly above 50kDa, several of these were in excess of 100kDa. Fraction 2 had no material above 100kDa, most of the bands identified being below 70kDa and bands in fraction 3 were mainly between 70 and 14kDa, little material was observed between 70 and 100kDa, and nothing above that.

Ammonium sulphate partitioned fractions used for immunisation (see table 3.21) gave the following pattern upon electrophoresis. The 20% precipitate contained bands over a wide molecular weight range, including some bands in excess of 100kDa, and this was also true of the 35% precipitate; bands between 80 and 110kDa being particularly well stained. The 45% precipitate contained

Figure 3.4 Molecular Weight Distribution of Proteins/Polypeptides in Crude Tick Extracts and Their Fractions after Electrophoretic Separation in SDS-Polyacrylamide Gels under Denaturing Conditions.

Numbers to the right of lane T represent the molecular weights (kDa) of protein standards observed in lanes S and T.

3.4



little material in excess of 100kDa, a band of around 70kDa being particularly well stained; and the 45% supernate contained little material above 70kDa and nothing in excess of 100kDa, bands at 70, 40 and 14kDa being particularly well stained.

Salivary gland extract contained material covering a wide molecular weight range, most bands though, were below 100kDa. The gut extract ranged from below 80kDa to around 14kDa, bands at 70 and 14kDa being most strongly stained.

Extracts of *Am. hebraeum*, *Am. variegatum*, *Rh. evertsi* and *Bo. microplus* all had protein/polypeptides covering a wide range of molecular weights, including some material in excess of 100kDa; most bands, however were under 100kDa.

3.5 Identification of Antigens in Whole Tick Extracts and Their Fractions.

As has already been mentioned (see section 3.3.4), the 45% ammonium sulphate supernate fraction and SEHPLC fraction 2 both protected guinea pigs to a significantly greater degree ($P < 0.05$) than all other fractions in a given experiment. As neither of these two fractions appeared to contain material in excess of 100kDa molecular weight (as determined by SDS-PAGE in section 3.4) all electrophoresis in this section was carried out in homogeneous 12% polyacrylamide gels.

The results presented here do not constitute an exhaustive analysis of all the protein/polypeptide species identified, but represent an attempt at identifying important/relevant antigens. In general there was little background staining, and only a minimal amount of non-specific staining of bands (few false positives). The process of photographic reproduction has enhanced several bands in some figures, whilst in other figures some bands appear fainter than in the original blots. Analyses are based on the banding observed in the original immunostained blots and not on their photographic copies.

3.5.1 Electrophoresis and Western Blotting of Whole Extracts.

3.5.1.1 Whole *Rh. appendiculatus* Tick Extracts Probed with Polyspecific Serum from *Rh.* *appendiculatus* Infested Guinea Pigs.

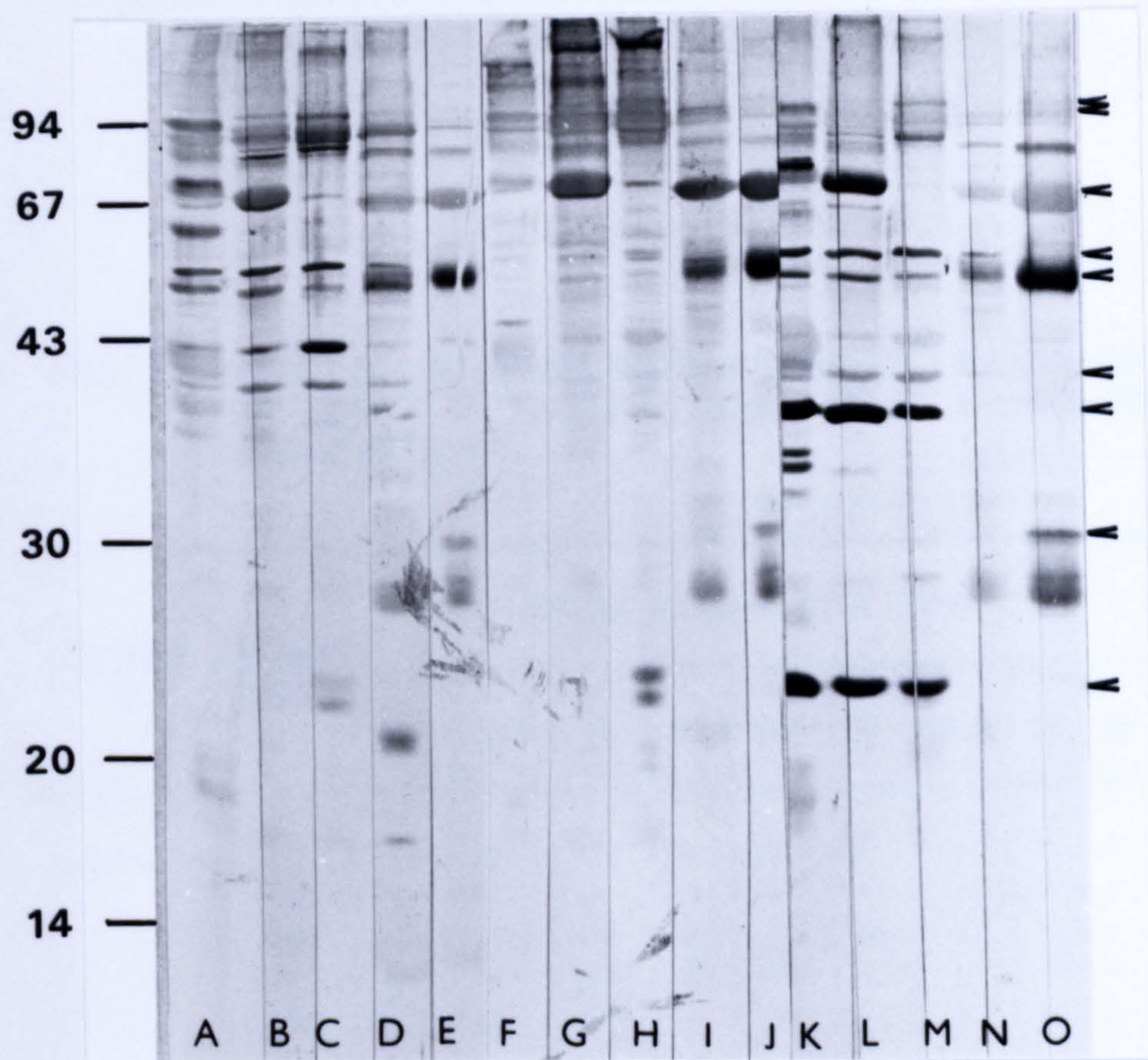
Whole extracts of unfed *Rh. appendiculatus* larvae, nymphs and adults (ULE, UNE and UAdE), and of partially fed (6 days) and fully fed females (PFFE and FFFE) were separated on SDS-polyacrylamide gels by electrophoresis and electroblotted onto nitrocellulose paper as in 2.7 and 2.8. The nitrocellulose sheets were then processed as in 2.9, using naive (normal) guinea pig serum, guinea pig antilarval serum (2 sequential infestations with *Rh. appendiculatus* larvae), guinea pig antinymphal serum (2 sequential infestations with *Rh. appendiculatus* nymphs) or guinea pig antiadult serum (2 sequential infestations with *Rh. appendiculatus* adults) (figure 3.5.1.1).

Each of the sera identified at least six discrete bands in each extract and in several cases in excess of 20 bands were recognised. Bands of 97.5 and 93.5kDa were weakly labelled in each of the five extracts by all three of the antisera. An antigenic species was identified at 70kDa in all five extracts when probed with antilarval serum; this was present as a strongly labelled diffuse band in UNE, PFFE and FFFE, whilst in the ULE and UAdE, a sharper clearly labelled band was identified. When probed with antinymphal or antiadult sera the 70kDa species was observed in UNE, PFFE and FFFE (always as a clearly or strongly labelled, diffuse band), but not in UAdE or ULE. A band of 55kDa was identified in the three extracts of unfed ticks probed by each of the three different antisera. The 55kDa band was still visible in PFFE, but was only weakly labelled; it was not identified in FFFE. Another antigenic band at 51.5kDa was positively labelled by all three antisera in each of the five extracts, but it appeared more strongly labelled in PFFE and FFFE than in the extracts of unfed ticks. A clearly labelled band at 40kDa was recognised by antilarval serum in ULE and UNE; it appeared weaker in UAdE and was not recognised in FFFE. Antinymphal serum did not identify this band in any of the five extracts, but antiadult serum clearly located the band in the three unfed tick extracts; it was weaker in PFFE and was not observed in FFFE. Antigenic species of 36.5kDa and 23kDa were present as intensely stained diffuse bands in each of the three unfed tick extracts when probed with antiadult serum, but they were not observed in the fed ticks. In addition, neither of these

Figure 3.5.1.1 Identification of Antigens in *Rh. appendiculatus* Tick Extracts using Polyspecific Serum from *Rh. appendiculatus* Guinea Pigs. Lanes A,F,K unfed larval extract (ULE); B,G,L unfed nymphal extract (UNE); C,H,M unfed adult extract (UAdE); D,I,N partially fed female extract (PFFE); E,J,O fully fed female extract (FFFE). A-E probed with antilarval (2 sequential infestations with *Rh. appendiculatus* larvae) serum; F-J probed with antinymphal (2 sequential infestations with *Rh. appendiculatus* nymphs) serum; K-O probed with antiadult (2 sequential infestations with *Rh. appendiculatus* adults). Bands referred to in text are arrowed to the right of lane O. Numbers to the left of lane A represent the molecular weights (kDa) of protein standards (94 phosphorylase b; 67 albumin; 43 ovalbumin; 30 carbonic anhydrase; 20 trypsin inhibitor; 14 alpha-lactalbumin).

Figure 3.5.1.2 Identification of Antigens in *Rh. appendiculatus* Tick Extracts using Post Immunisation Guinea Pig Sera. Lanes A,F,K unfed larval extract (ULE); B,G,L unfed nymphal extract (UNE); C,H,M unfed adult extract (UAdE); D,I,N partially fed female extract (PFFE); E,J,O fully fed female extract (FFFE). A-E probed with antiULE serum; F-J probed with antiUNE serum; K-O probed with antiUAdE serum. Bands referred to in text are arrowed to the right of lane O. Numbers to the left of lane A represent the molecular weights (kDa) of protein standards (listed in Figure 3.5.1.1).

3.5.1.1



3.5.1.2

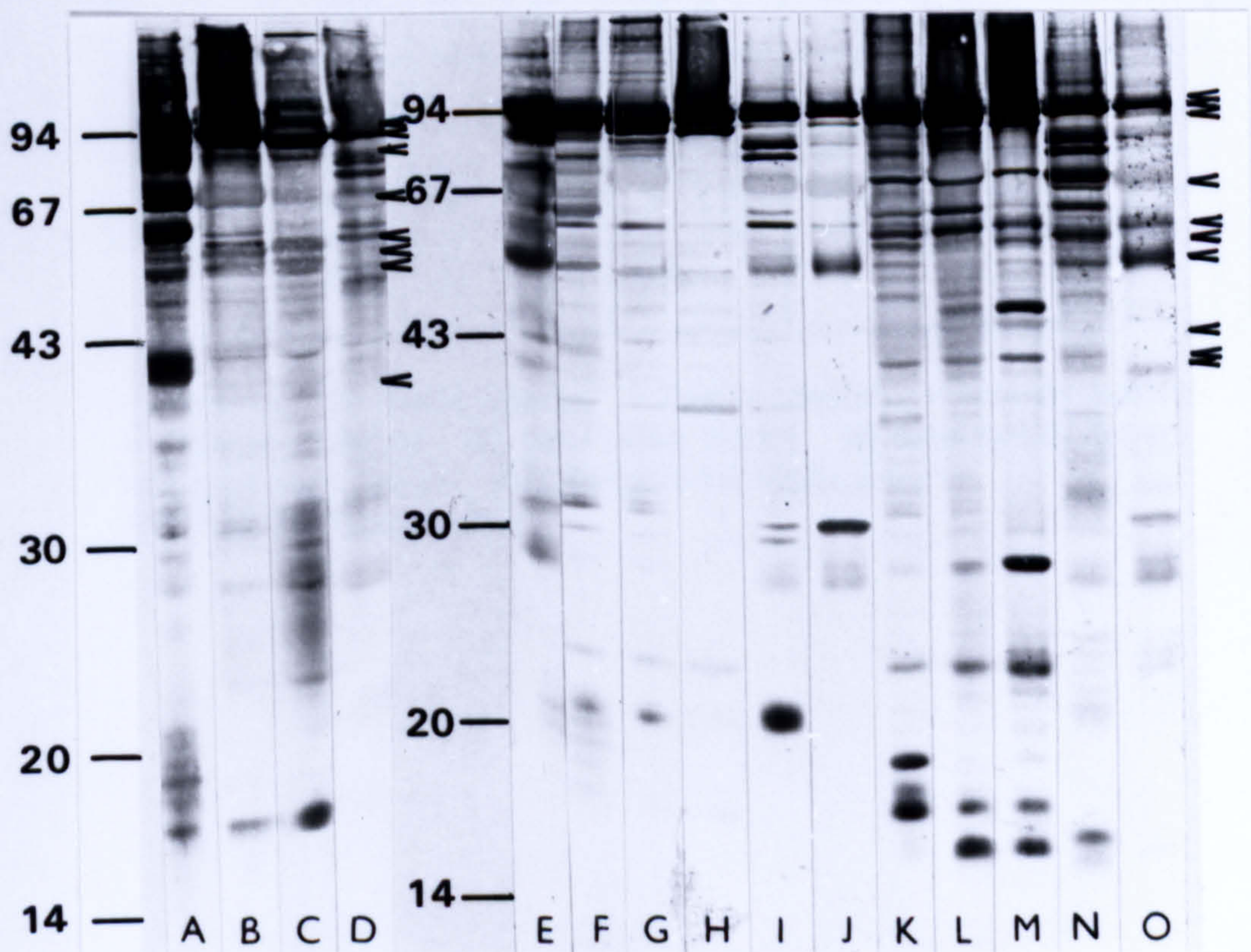


TABLE 3.3 SUMMARY OF MAJOR ANTIGENS IDENTIFIED IN WHOLE RH. APPENDICULATUS TICK EXTRACTS BY POLYSPECIFIC SERUM FROM RH. APPENDICULATUS INFESTED GUINEA PIGS.

ANTISERUM TO	ANTIGEN SOURCE	ANTIGEN MOLECULAR WEIGHT (kDa)								
		97.5	93.5	70	55	51.5	40	36.5	30	23
Larvae	ULE	+/-	+/-	+/-	++	+	+	-	-	-
	UNE	+/-	+/-	++	++	+	+	-	-	-
	UAdE	+/-	+/-	+/-	++	+	+/-	-	-	-
	PFFE	+/-	+/-	+/>++	+/-	+/>++	-	-	-	-
	FFFE	+/-	+/-	+/>++	-	++	-	-	+	-
Nymphs	ULE	+/-	+/-	-	+/-	+/-	-	-	-	-
	UNE	+/-	+/-	++	+/-	+/-	-	-	-	-
	UAdE	+/-	+/-	-	+/-	+/-	-	-	-	-
	PFFE	+/-	+/-	++	-	+	-	-	-	-
	FFFE	+/-	+/-	++	-	++	-	-	+	-
Adults	ULE	+/-	+/-	-	++	+	+	+++	-	+++
	UNE	+/-	+/-	+++	++	+	+	+++	-	+++
	UAdE	+/-	+/-	-	++	+	+	+++	-	+++
	PFFE	+/-	+/-	+	+/-	+	+/>+	-	-	-
	FFFE	+/-	+/-	+/>++	-	++	-	-	+	-

- Unlabelled +/- Weakly labelled + Clearly labelled ++ Strongly labelled
 +++ Intensely labelled ULE Unfed Larval Extract UNE Unfed Nymphal Extract
 UAdE Unfed Adult Extract PFFE Partially Fed Female Extract
 FFFE Fully Fed Female Extract

TABLE 3.4 SUMMARY OF MAJOR ANTIGENS IDENTIFIED IN WHOLE *RH. APPENDICULATUS* EXTRACTS BY POLYSPECIFIC POST IMMUNISATION GUINEA PIG SERA.

ANTISERUM TO	ANTIGEN SOURCE	ANTIGEN MOLECULAR WEIGHT (kDa)									
		97.5	93.5	84	70	60	55	51.5	40	36.5	23
ULE	ULE	+++	+++	+++	++	+	+/-	+	-	-	-
	UNE	+++	+++	+++	+	+	+/-	+	-	-	-
	UAdE	+++	+++	+++	+/-	+	+/-	+	-	-	-
	PFFE	++	++/+++	+	+	+	+/-	+	-	-	-
	FFFE	+	++	+/-	+	+	-	++	-	-	-
UNE	ULE	+++	+++	+++	+/-	+	+/-	+	-	-	-
	UNE	+++	+++	+++	+	+	+/-	+	-	-	-
	UAdE	+++	+++	+++	+/-	+	+/-	+	-	-	-
	PFFE	+++	+++	+	+	+	+/-	+	-	-	-
	FFFE	++/+++	++/+++	+	+	+	-	++	-	-	-
UAdE	ULE	+++	+++	+++	++	+	+/-	+	+	-	-
	UNE	+++	+++	+++	++	+	+/-	+	+	-	-
	UAdE	+++	+++	+++	++	+	+/-	+	+	-	-
	PFFE	+/++	+++	+/-	++	+	+/-	+/++	+/-	-	-
	FFFE	+/-	++	+/-	+	+	-	++	-	-	-

- Unlabelled +/- Weakly labelled + Clearly labelled ++ Strongly labelled
+++ Intensely labelled ULE Unfed Larval Extract UNE Unfed Nymphal Extract
UAdE Unfed Adult Extract PFFE Partially Fed Female Extract FFFE Fully Fed Female Extract

bands were identified in any of the five extracts when probed with antilarval or antinymphal sera. A singular 30kDa species was identified in FFFE probed by all the three antisera; this band was not seen in any of the other extracts (table 3.3).

Normal guinea pig serum failed to identify any of these antigen species.

3.5.1.2 Whole *Rh. appendiculatus* Tick Extracts Probed with Post Immunisation Guinea Pig Sera.

When the same five tick extracts were probed with antisera raised by inoculation with ULE, UNE or UAdE (figure 3.5.1.2) each of the sera identified at least 10 bands in each extract and in several cases in excess of 30 antigenic bands were recognised. Intensely labelled bands at 97.5, 93.5 and 84kDa were identified in all the three extracts of unfed ticks by all three antisera. Although still clearly labelled, staining was not so strong in PFFE and was even less strong in FFFE. A 70kDa species present as a diffuse band was identified in all five of the extracts by each of the antisera. A 60kDa antigen species and a 51.5kDa species were clearly identified in all five extracts by each of the three antisera; the latter of the two bands was more strongly stained in FFFE than in the unfed tick extracts. As with extracts probed with post infestation sera (see section 3.5.1.1), a band of 55kDa was observed in each of the three unfed tick extracts when probed with all the three antisera. It was weaker in PFFE and absent in the FFFE. A band of 40kDa was clearly identified in ULE, UNE and

UAdE by antiUAdE only; it was not recognised by antiULE or antiUNE in any of the five extracts, or by any of the three sera in the PFFE and FFFE. The 36.5kDa and 23kDa species identified in extracts of unfed ticks when probed with post adult infestation serum (see section 3.5.1.1), were not recognised by any of the three immunisation antisera (table 3.4).

Normal guinea pig serum did not recognise any of these antigens.

3.5.1.3. Whole *Rh. appendiculatus* Salivary Gland Extract Probed with Guinea Pig Post Infestation (Adult Male and Female) Serum and Guinea Pig Post Immunisation Serum

Salivary gland extract from partially fed females (see 2.2.3) was probed with guinea pig antiadult (2 sequential infestations with *Rh. appendiculatus* adults) serum or with guinea pig antiUNE/ammonium sulphate 45% supernate serum (anti45supt serum) (table 3.21).

In excess of 10 antigenic bands were recognised by antiadult serum. These included intensely stained bands at 140, 97.5 and 93.5kDa. Bands at 84, 70, 60, 55 and 51.5kDa were all recognised as were several weakly labelled bands, including ones at 36.5 and 23kDa (figure 3.5.1.3).

Only four discrete bands were identified in the extract by the anti45supt serum, these being at 97.5, 93.5, 70 and 60kDa. The

97.5kDa band was strongly, and the 93.5 and 70kDa species intensely stained. The 70kDa band was fairly diffuse, whilst the 60kDa band was less strongly labelled (table 3.5).

None of these antigens were recognised by normal guinea pig serum.

3.5.1.4 *Rh. appendiculatus* Gut Extract Probed with Guinea Pig Post Infestation (Adult Male and Female) Serum and Guinea Pig Post Immunisation Serum.

Gut extract from partially fed females was probed with guinea pig antiadult (2 sequential infestations with *Rh. appendiculatus* adults) serum or with guinea pig anti45supt serum.

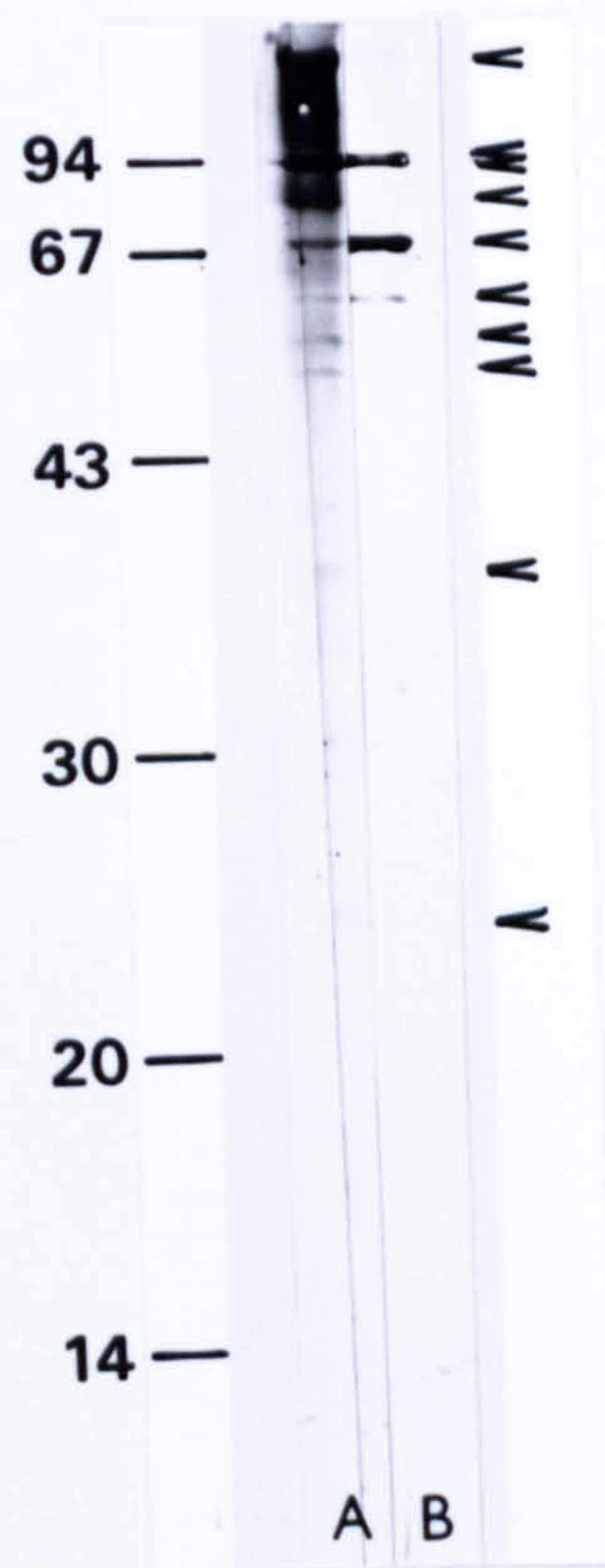
The antiadult serum identified antigens at 84, 70, 60, 53 and 27Da (figure 3.5.1.4). Of these, the 84kDa band was weakly labelled, whilst the 70kDa species was more clearly labelled, but was diffuse. A stronger band was seen at 60kDa and a strongly stained diffuse band was observed at 55kDa. A clearly labelled diffuse band was observed at 27kDa. No bands were located at 36.5 and 23kDa. Probing the gut extract with the anti45supt serum revealed a very weakly staining band at 97.5kDa and another weakly labelled band at 93.5kDa. This serum also recognised the 84kDa species as a weakly stained band. The 70kDa species was diffuse and clearly stained whilst the 60kDa antigen was very intensely labelled. As with the antiadult serum a strong diffuse

Figure 3.5.1.3 Identification of Antigens in *Rh. appendiculatus* Salivary Gland Extract using Guinea Pig Post Infestation (Adult Male and Female) Serum and Guinea Pig Post Immunisation Serum. Lane A salivary gland extract (SGE) probed with antiadult (2 sequential infestations with *Rh. appendiculatus* adults) serum; B SGE probed with anti45supt serum (UNE fraction). Bands referred to in text are arrowed to the right of lane B. Numbers to the left of lane A represent the molecular weights (kDa) of protein standards (listed in Figure 3.5.1.1).

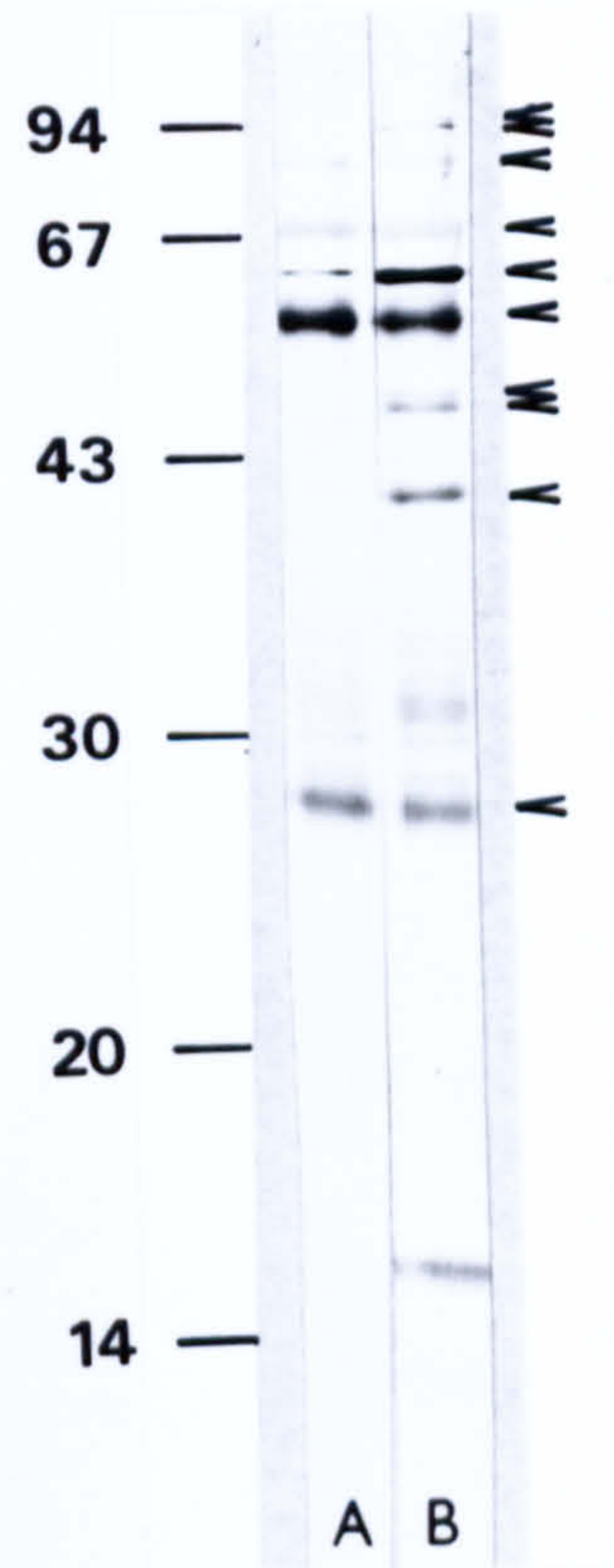
Figure 3.5.1.4 Identification of Antigens in *Rh. appendiculatus* Gut Extract using Guinea Pig Post Infestation (Adult Male and Female) Serum and Guinea Pig Post Immunisation Serum. Lane A gut caecae extract (GCE) probed with antiadult (2 sequential infestations with *Rh. appendiculatus* adults) serum; B GCE probed with anti45supt serum (UNE fraction). Bands referred to in text are arrowed to the right of lane B. Numbers to the left of lane A represent the molecular weights (kDa) of protein standards (listed in Figure 3.5.1.1).

Figure 3.5.2.1 Identification of Antigens in Fractions of *Rh. appendiculatus* Unfed Larval Extract using Guinea Pig Post Infestation (Adult Male and Female) Serum and Guinea Pig Post Immunisation Serum. Lanes A,B unfed larval extract; C,D ULE, charcoal treated; E,F ULE, dialysed; G,H ULE, PEG 8000 precipitate; K,L ULE, ammonium sulphate precipitate; K,L ULE, ammonium sulphate supernate. A,C,E,G,I,K probed with antiadult (2 sequential infestations with *Rh. appendiculatus* adults) serum; B,D,F,H,J,L probed with anti45supt serum (UNE fraction). Bands referred to in text are arrowed to the right of lane L. Numbers to the left of lane A represent the molecular weights (kDa) of protein standards (listed in Figure 3.5.1.1).

3.5.1.3



3.5.1.4



3.5.2.1

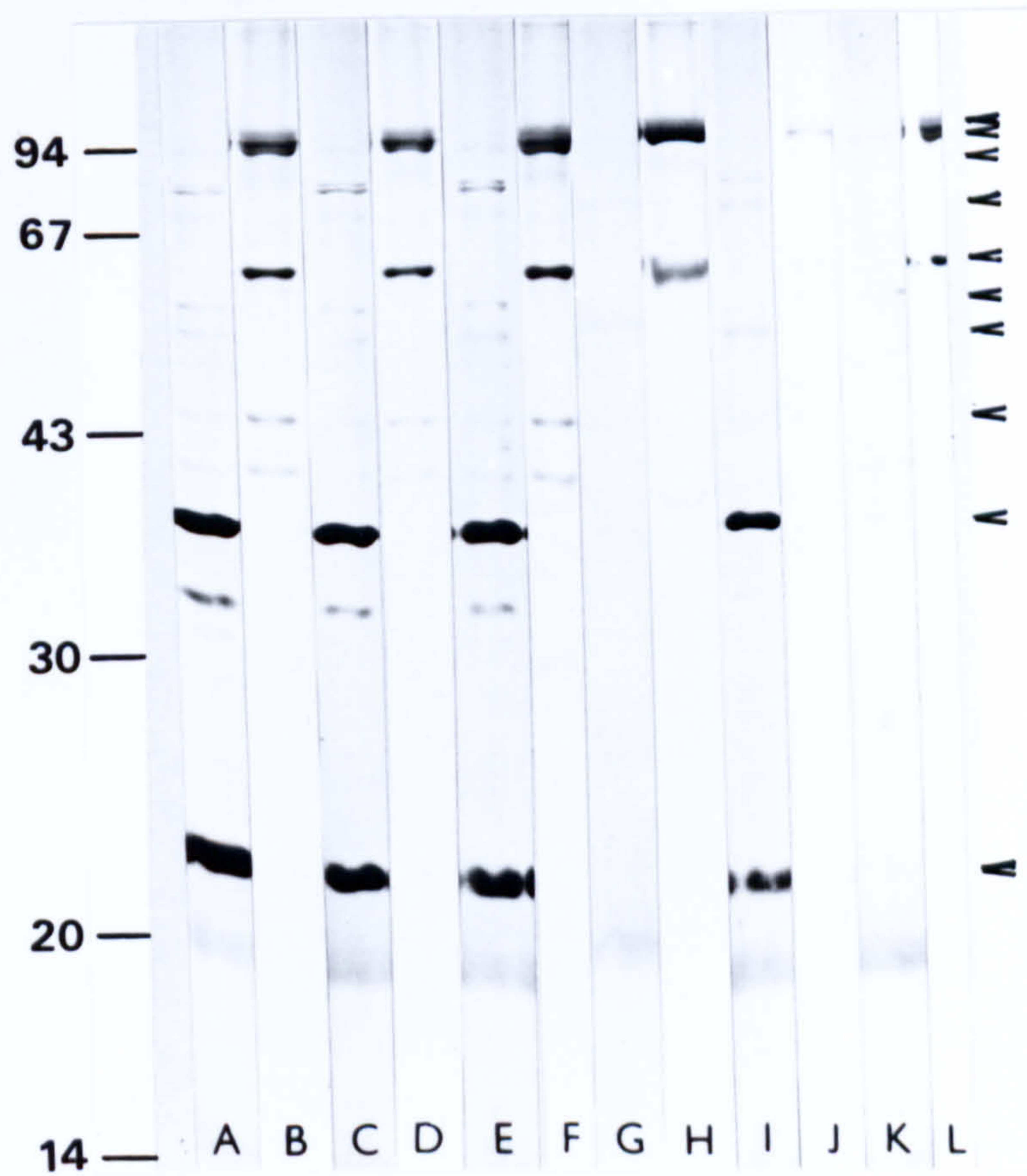


TABLE 3.5 SUMMARY OF MAJOR ANTIGENS IDENTIFIED IN *RH. APPENDICULATUS* SALIVARY GLAND EXTRACT BY ANTIADULT AND ANTI45SUPT SERA.

ANTIGEN MOLECULAR WEIGHT (kDa)	ANTISERUM	
	Antiadult	Anti45supt
140	+++	-
97.5	+++	++
93.5	+++	+++
84	+ / ++	-
70	+ / ++	+++
60	+	+
55	+	-
51.5	+	-
36.5	+ / -	-
23	+ / -	-

- Unlabelled +/- Weakly labelled
+ Clearly labelled ++ Strongly labelled
+++ Intensely labelled

TABLE 3.6 SUMMARY OF MAJOR ANTIGENS IDENTIFIED IN FEMALE *RH. APPENDICULATUS* GUT CAECAE EXTRACT.

ANTIGEN MOLECULAR WEIGHT (kDa)	ANTISERUM	
	Antiadult	Anti45supt
97.5	-	+/-
93.5	-	+/-
84	+/-	+/-
70	+	+
60	+/++	+++
55	++	++
46	-	+/-
45	-	+
40	-	+/++
36.5	-	-
27	+	+
23	-	-

- Unlabelled +/- Weakly labelled
+ Clearly labelled ++ Strongly labelled
+++ Intensely labelled

TABLE 3.7 SUMMARY OF MAJOR ANTIGENS IDENTIFIED IN RH. APPENDICULATUS UNFED LARVAL EXTRACT AFTER VARIOUS TREATMENTS.

ANTISERUM	ANTIGEN SOURCE	ANTIGEN MOLECULAR WEIGHT (kDa)									
		97.5	93.5	84	70	60	55	51.5	40	36.5	23
Antiadult	ULE	+/-	+/-	-	+/-	-	+	+	+	+++	+++
	ULE, charcoal treated	+/-	+/-	-	+/-	-	+	+	+	+++	+++
	ULE, dialysed	+/-	+/-	-	+/-	-	+	+	+	+++	+++
	ULE, PEG 8000 supernate	-	-	-	+/-	-	-	-	-	-	-
	ULE, ammonium sulphate precipitate	-	-	-	+/-	-	-	+/-	-	+	+
	ULE, ammonium sulphate supernate	-	+/-	-	-	-	+/-	-	+/-	-	-
Anti45supt	ULE	++	++	+/-	-	++	-	-	+	-	-
	ULE, charcoal treated	++	++	+/-	-	++	-	-	+	-	-
	ULE, dialysed	++	++	+/-	-	++	-	-	+	-	-
	ULE, PEG 8000 supernate	++	++	+/-	-	++	-	-	+/-	-	-
	ULE, ammonium sulphate precipitate	+	+	-	-	+	-	-	-	-	-
	ULE, ammonium sulphate supernate	++	++	+/-	-	+++	-	-	+	-	-

- Unlabelled +/- Weakly labelled + Clearly labelled ++ Strongly labelled
 +++ Intensely labelled ULE Unfed Larval Extract

antigenic band was identified at 55kDa. The anti45supt serum also revealed antigenic bands at 46, 45 and 40kDa in addition to one at 27kDa (table 3.6).

Normal serum failed to recognise any of these bands.

3.5.2 Electrophoresis and Western Blotting of Fractionated /Purified *Rh. appendiculatus* Tick Extracts.

3.5.2.1 Unfed Larval Extract after Various Treatments.

ULE after various treatments (see 2.3.1 - 2.3.4), was probed with guinea pig antiadult (2 sequential infestations with *Rh. appendiculatus* adults) serum or with guinea pig anti45supt serum (table 3.21).

The antigen profiles for unfractionated ULE, charcoal treated ULE and dialysed ULE (figure 3.5.2.1) were all similar to the ULE profiles obtained in 3.5.1.1 and 3.5.1.2.

Antiadult serum failed to recognise any antigens in the ULE PEG 8000 supernate other than a very faint band at 70kDa. The anti45supt serum however located sharp bands at 97.5kDa, 93.5kDa, a strongly stained diffuse band at 60kDa and a very weak band at 40kDa. The 70kDa antigen band was not recognised.

Antiadult serum recognised bands at 36.5kDa and 23kDa in the ammonium sulphate precipitate, but neither of these bands were recognised in the ammonium sulphate supernate. The anti45supt

serum identified antigens at 97.5kDa, 93.5kDa and 60kDa in both the precipitate and supernate, though in each case the bands were stronger in the supernate. A 40kDa species was recognised in the supernate only, but the 70kDa band was not identified in either supernate or precipitate (table 3.7).

3.5.2.2 HPLC Size-Exclusion Fractionation of *Rh. appendiculatus* Unfed Nymphal Extract.

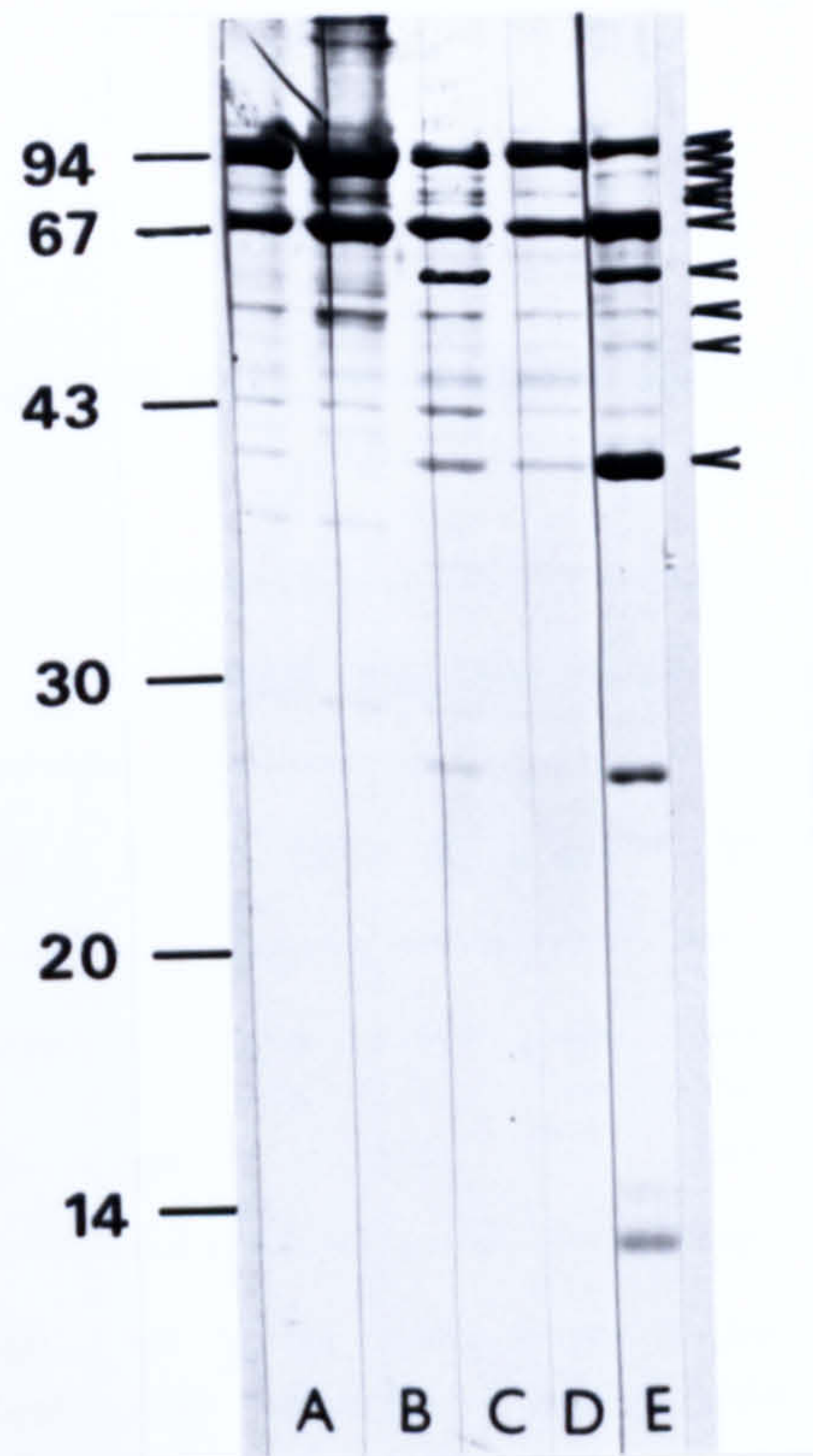
SEHPLC fractions and their whole parent extract were probed with serum from guinea pigs immunised with fraction 2 (antiF2 serum) in table 3.19 (figure 3.5.2.2a).

The antiF2 serum revealed some antigenic material in excess of 100kDa in fraction 1. The 97.5 and 93.5kDa bands were intensely stained as was a broad band at 70kDa. Clearly labelled bands were also observed at 84, 77.5, 75 and 55kDa; the 60kDa species was only weakly stained. Fraction 2 had antigens strongly labelled at 97.5, 93.5 and 70kDa, and clearly labelled at 84, 77.5, 75 and 55kDa. In addition, a band at 60kDa was intensely stained, and a very weakly stained band at 51.5kDa was also observed. A 40kDa species was clearly labelled in this fraction. The antiF2 serum recognised strongly labelled bands in fraction 3 at 97.5, 93.5 and 70kDa. The 84kDa band was weakly labelled and labelling of the 77.5kDa band was even weaker. The 75kDa band was not identified at all, neither was the 60kDa antigen, but a weak band was seen at 55kDa. The 40kDa band was patent, but was less strongly stained than in fraction 2.

Figure 3.5.2.2a Identification of Antigens in Size-Exclusion Fractions (1,2,3) of *Rh. appendiculatus* Unfed Nymphal Extract using Guinea Pig Post Immunisation Sera. Lane A unfed nymphal extract (UNE); B UNE SEHPLC fraction 1; C,E UNE SEHPLC fraction 2; D UNE SEHPLC fraction 3. A-D probed with antifraction 2 serum; E probed with anti45supt serum (UNE fraction). Bands referred to in text are arrowed to the right of lane E. Numbers to the left of lane A represent the molecular weights (kDa) of protein standards (listed in Figure 3.5.1.1).

Figure 3.5.2.2b Identification of Antigens in Size-Exclusion Fractions (1,2,3) of *Rh. appendiculatus* Unfed Nymphal Extract using Guinea Pig Post Immunisation-Post Infestation (Adult Males and Females) Sera. Lanes A,D,G unfed nymphal extract (UNE) SEHPLC fraction 1; B,E,H UNE SEHPLC fraction 2; C,F,I UNE SEHPLC fraction 3. A-C probed with antifraction 2 serum obtained after one infestation with *Rh. appendiculatus* adults; D-F probed with antiF2 serum after 2 adult infestations; G-I probed with antiF2 serum after 3 adult infestations. Bands referred to in text are arrowed to the right of lane I. Numbers to the left of lane A represent the molecular weights (kDa) of protein standards (listed in Figure 3.5.1.1).

3.5.2.2



a

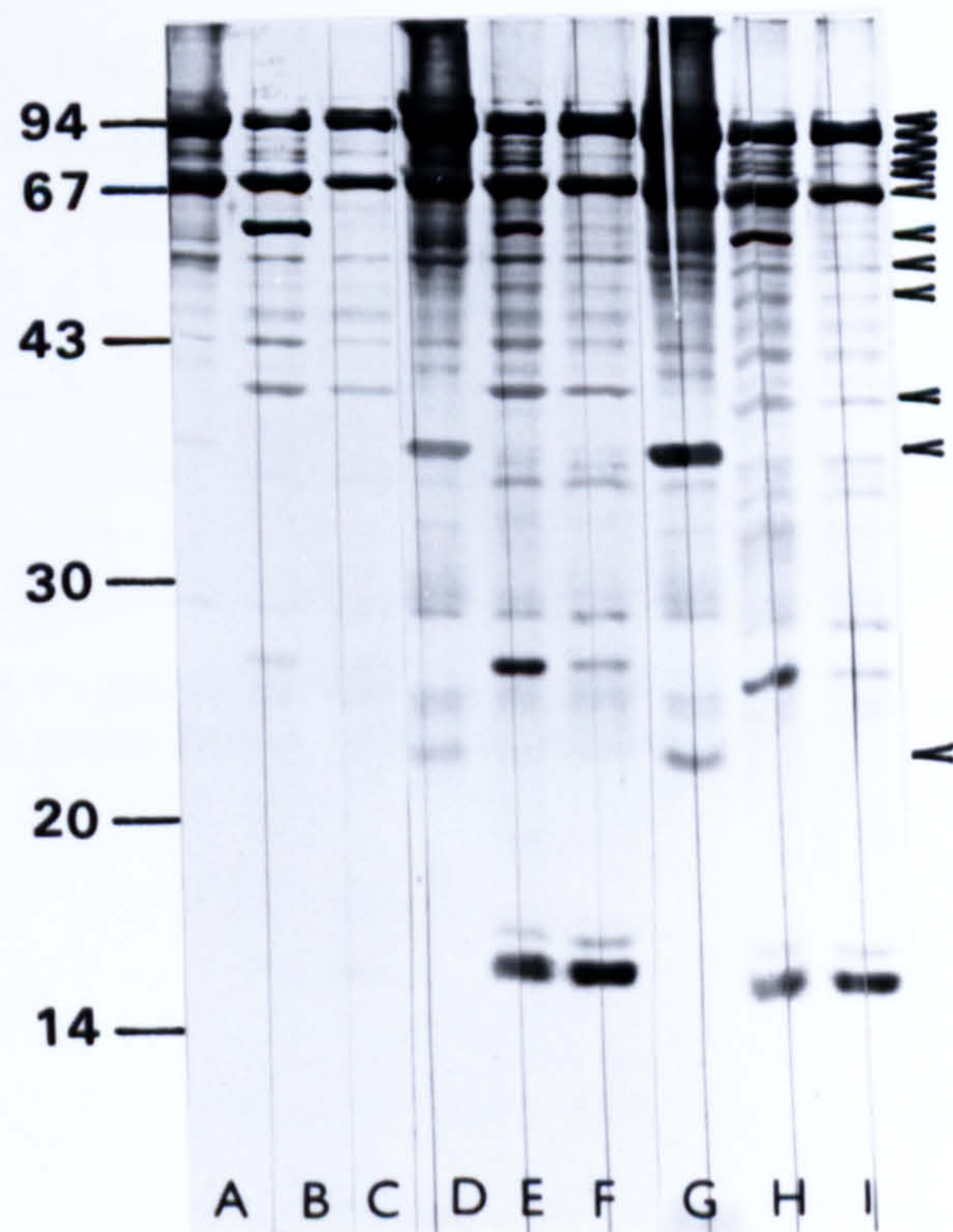
**b**

TABLE 3.8 SUMMARY OF MAJOR ANTIGENS IDENTIFIED IN *RH. APPENDICULATUS* UNFED NYMPHAL EXTRACT FRACTIONATED BY SEHPLC.

ANTISERUM	SEHPLC FRACTION	ANTIGEN MOLECULAR WEIGHT (kDa)											
		97.5	93.5	84	77.5	75	70	60	55	51.5	40	36.5	23
AntiF2	1	+++	+++	+ / ++	+	+	+++	+ / -	+ / ++	-	-	-	-
	2	++	++	+	+	+	++	+++	+	+ / -	+ / ++	-	-
	3	++	++	+ / -	+ / +	-	++	-	+ / -	-	+	-	-
AntiF2 + 3 adult feeds	1	+++	+++	+++	++	++	+++	+ / -	++	+	-	+++	++
	2	++	++	+ / ++	+ / ++	+ / ++	++	+++	+ / ++	+ / ++	+ / ++	-	-
	3	++	++	+ / -	+ / +	-	++	-	+	+	+	-	-
AntiF1	1	+++	+++	-	-	-	++	-	-	-	-	-	-
AntiF3	3	+ / -	++	-	-	-	+ / ++	-	-	-	+ / -	-	-
Anti45supt	2	+++	+++	+ / -	-	-	+++	+++	+	+	+++	-	-
AntiF2	1a	+++	+++	+ / ++	++	++	+++	-	-	-	-	-	-
	2a	++	++	+	+	-	+++	-	+	-	+ / -	-	-
	3a	++	++	+ / -	+ / +	-	++	+ / -	+ / -	-	+ / -	-	-
Anti45supt	2a	++	-	+	-	-	+++	+	+	+ / -	+++	-	-
	3a	+	-	-	-	-	++	++	-	-	++	-	-

- Unlabelled + / - Weakly labelled + Clearly labelled ++ Strongly labelled
+++ Intensely labelled UNE Unfed Nymphal Extract SEHPLC Size Exclusion HPLC

When the fractions were probed with antiF2 serum, bands at 36.5 and 23kDa were not identified; however, serum from the same guinea pig obtained after additional one, two or three sequential infestations with *Rh. appendiculatus* adults, detected both of these bands in increasing intensity from one through to three infestations in fraction 1 (figure 3.5.2.2b). The 36.5 and 23kDa species were not identified in fractions 2 and 3.

Upon probing fraction 1 with its own antiserum several bands in excess of 100kDa were identified. The 97.5 and 93.5kDa bands were intensely stained as was to a lesser extent the 70kDa species. The 60 and 40kDa species were not identified. Fraction 2 probed with antiF2 serum revealed an antigen profile identical to that mentioned earlier, with bands labelled at 97.5, 93.5, 84, 77.5, 75, 70, 60, 55, 51.5 and 40kDa. When fraction 3 was probed with its own antiserum bands were identified at 93.5 and 70kDa along with a weakly staining band at 40kDa and a very weak band at 97.5kDa. No other bands were identified (figure 3.5.2.2c).

When fraction 2 was probed with anti45supt serum the 97.5, 93.5, 70, 60 and 40kDa species were all intensely labelled. A weakly staining band was identified at 84kDa and bands at 55 and 51.5kDa were also labelled; the 77.5 and 75kDa species were not identified (figure 3.5.2.2a).

SEHPLC fractions from a different UNE (referred to as fractions 1a, 2a and 3a, of which fraction 3a induced a higher level of immunity than fraction 2a upon immunisation, see table 3.20),

were probed with antiF2 serum. Fraction 1a had an antigenic profile similar to that of fraction 1 in figure 3.5.2.2a except that bands at 60 and 55kDa were absent; whilst in fraction 2a the antiF2 serum failed to identify antigenic material at 75 and 60kDa, the profile being otherwise similar to that of fraction 2. All the antigens recognised in fraction 3 were identified in fraction 3a. Additionally, bands of 60 and 55kDa were observed in the latter, though they were only weakly labelled (figure 3.5.2.2d).

When fraction 2a was probed with anti45supt serum, bands of 97.5, 84, 70, 60, 55, 51.5 and 40kDa were identified. The 70 and 40kDa bands were particularly intense, but the 60kDa species, although clearly labelled, was less strong. In fraction 3a only the 97.5, 70, 60 and 40kDa species were recognised. The 97kDa band though clearly labelled, was not as strong as in fraction 2a, neither were the 70 and 40kDa bands as strongly labelled. The 60kDa band was more strongly labelled in fraction 3a than in fraction 2a (figure 3.5.2.2d).

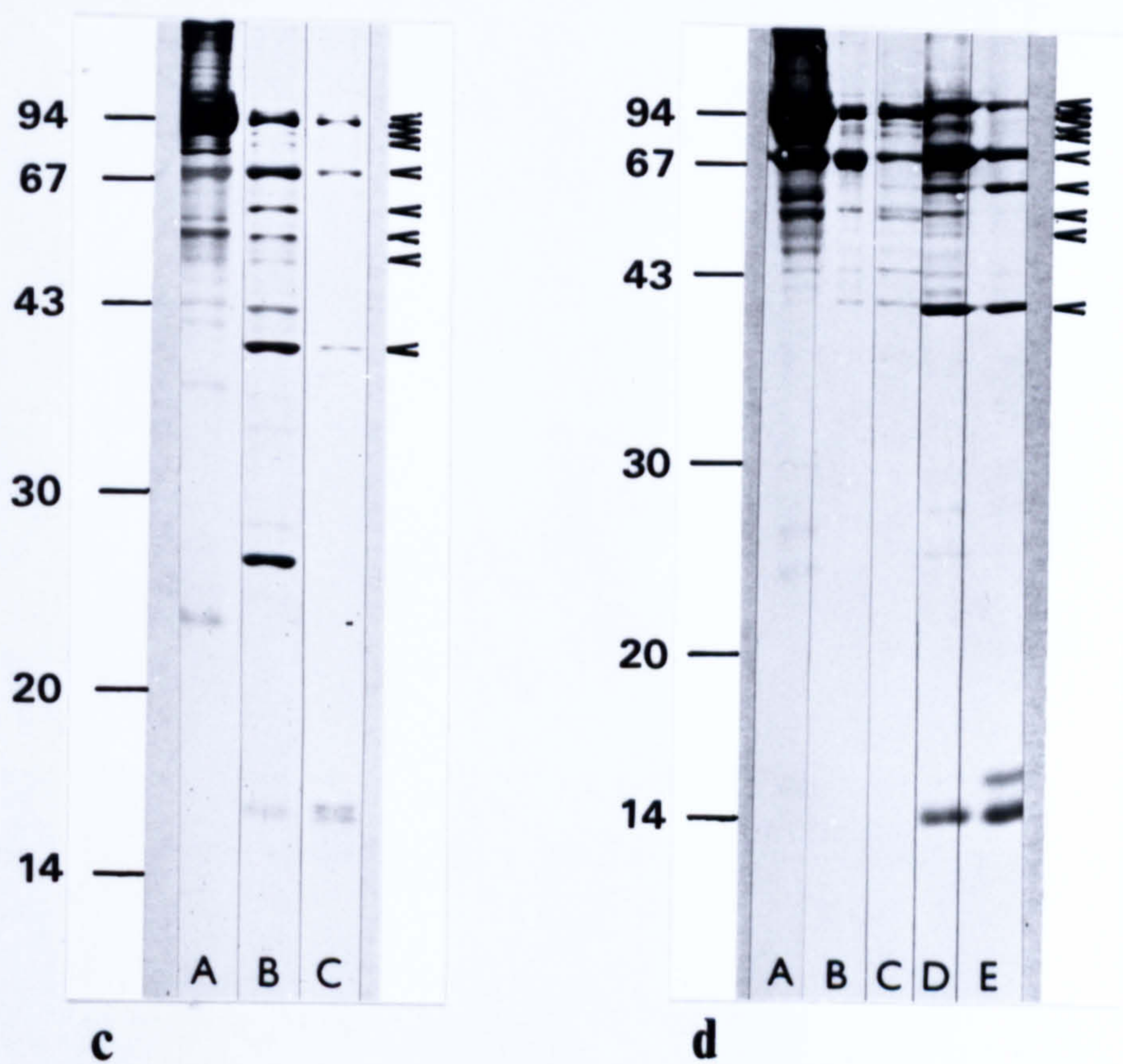
The location of the major antigens identified in this section are summarised in table 3.8.

Figure 3.5.2.2c Identification of Antigens in Size-Exclusion Fractions (1,2,3) of *Rh. appendiculatus* Unfed Nymphal Extract using Their Guinea Pig Post Immunisation Sera. Lane A unfed nymphal extract (UNE) SEHPLC fraction 1 probed with antifraction 1 serum; B UNE SEHPLC fraction 2 probed with antifraction 2 serum; C UNE SEHPLC fraction 3 probed with antifraction 3 serum. Bands referred to in text are arrowed to the right of lane C. Numbers to the left of lane A represent the molecular weights (kDa) of protein standards (listed in Figure 3.5.1.1).

Figure 3.5.2.2d Identification of Antigens in Size-Exclusion Fractions (1a,2a,3a) of *Rh. appendiculatus* Unfed Nymphal Extract using Guinea Pig Post Immunisation Sera. Lane A unfed nymphal extract (UNE) SEHPLC fraction 1a; B,C UNE SEHPLC fraction 2a; D,E UNE SEHPLC fraction 3a. A-C probed with antifraction 2 serum; D,E probed with anti45supt serum. Bands referred to in text are arrowed to the right of lane E. Numbers to the left of lane A represent the molecular weights (kDa) of protein standards (listed in Figure 3.5.1.1).

Figure 3.5.2.3a Identification of Antigens in Ammonium Sulphate Partitioned Fractions of *Rh. appendiculatus* Unfed Nymphal Extract using Guinea Pig Post Infestation (Adult Male and Female) Serum and Guinea Pig Post Immunisation Serum. Lanes A,B unfed nymphal extract (UNE) 20% ammonium sulphate (AS) precipitate fraction; C,D UNE 35%AS precipitate fraction; E,F UNE 45%AS precipitate fraction; G,H UNE 45%AS supernate fraction; I,J UNE. A,C,E,G,I probed with antiadult (2 sequential infestations with *Rh. appendiculatus* adults) serum; B,D,F,H,J probed with anti45supt serum. Bands referred to in text are arrowed to the right of lane J. Numbers to the left of lane A represent the molecular weights (kDa) of protein standards (listed in Figure 3.5.1.1).

3.5.2.2



3.5.2.3

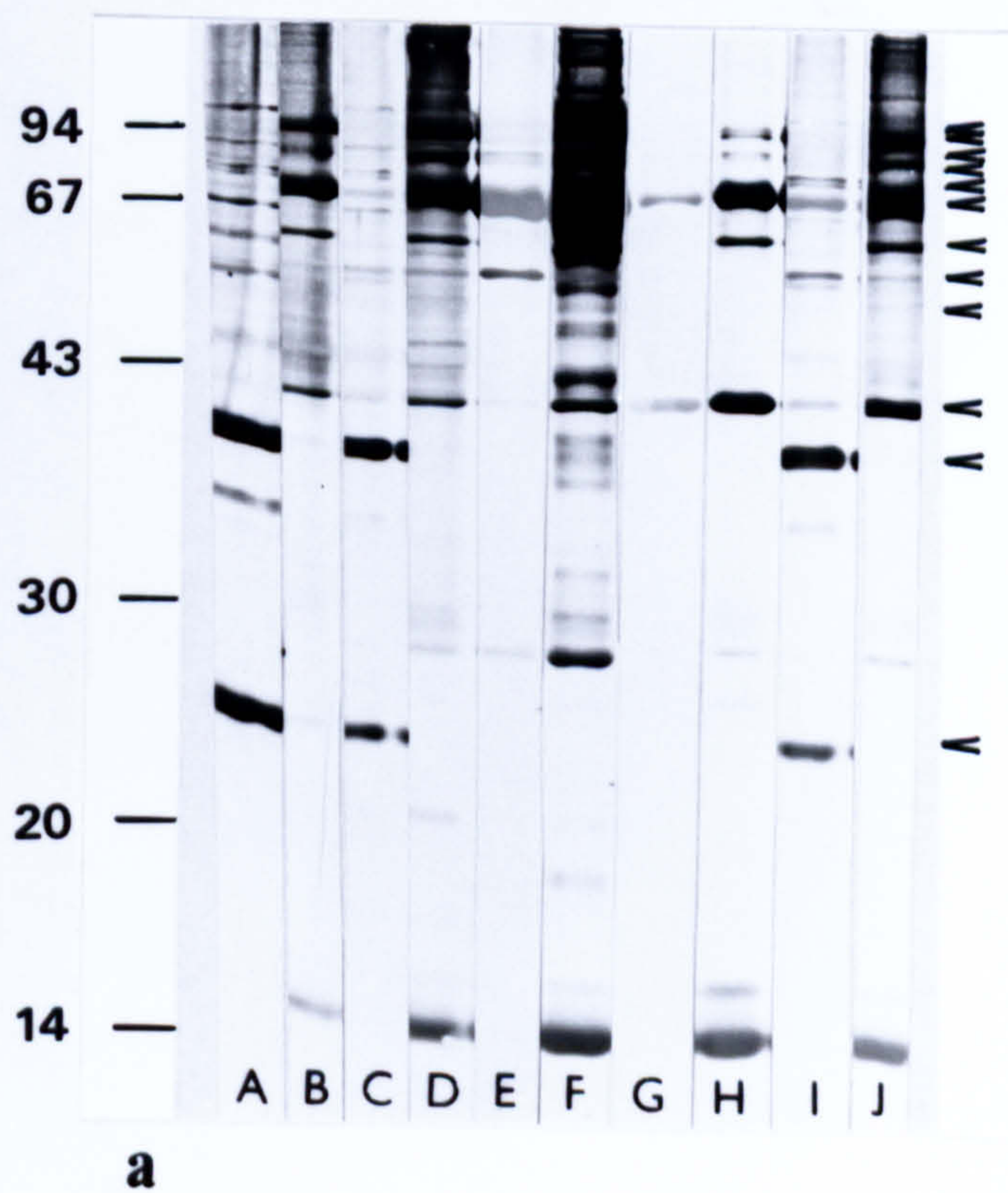


Figure 3.5.2.3b Identification of Antigens in the 45% Ammonium Sulphate Supernate Fraction of *Rh. appendiculatus* Unfed Nymphal Extract using Guinea Pig Post Immunisation (Anti45supt) Sera. Lanes A and B UNE 45% ammonium sulphate supernate fraction. A probed with anti45supt (135µg) serum; B probed with anti45supt (1350µg) serum. Bands referred to in text are arrowed to the right of lane B. Numbers to the left of lane A represent the molecular weights (kDa) of protein standards (listed in Figure 3.5.1.1).

Figure 3.5.2.3c Identification of Antigens in the 45% Ammonium Sulphate Supernate Fraction of *Rh. appendiculatus* Unfed Nymphal Extract using Guinea Pig Post Immunisation (AntiNCP-bound antigens) Serum. Lanes A and B UNE 45% ammonium sulphate supernate fraction. A probed with anti45supt serum; B probed with antiserum to nitrocellulose antigen-bearing particles. Bands referred to in text are arrowed to the right of lane B. Numbers to the left of lane A represent the molecular weights (kDa) of protein standards (listed in Figure 3.5.1.1).

3.5.2.3

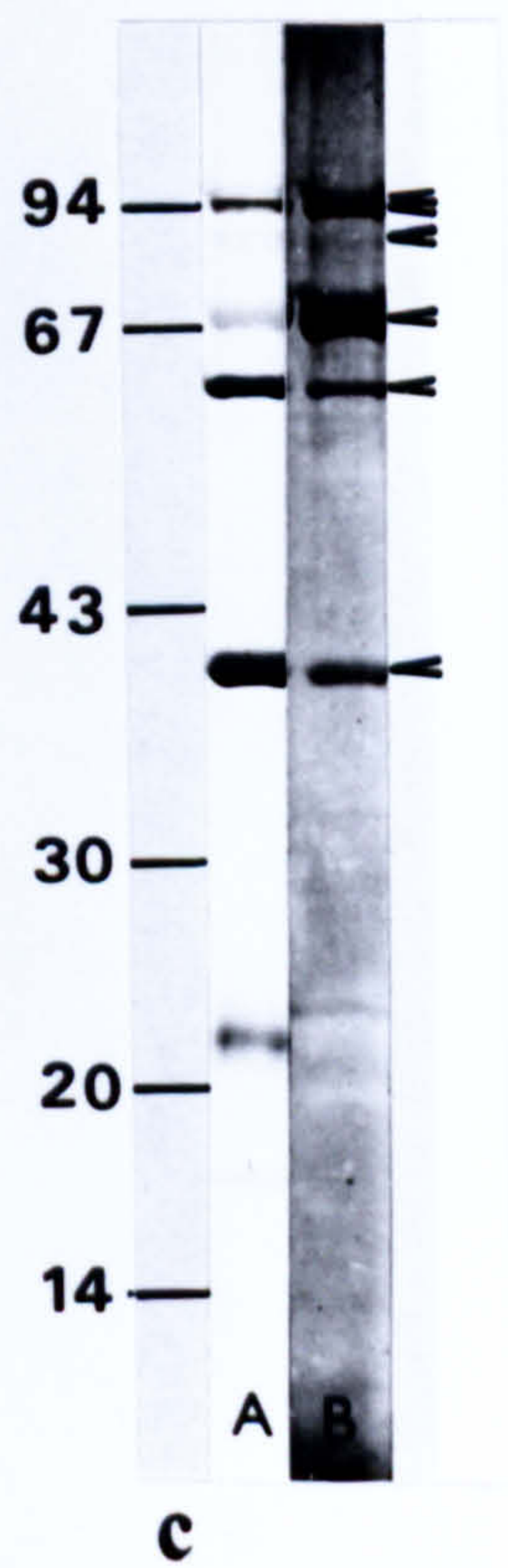
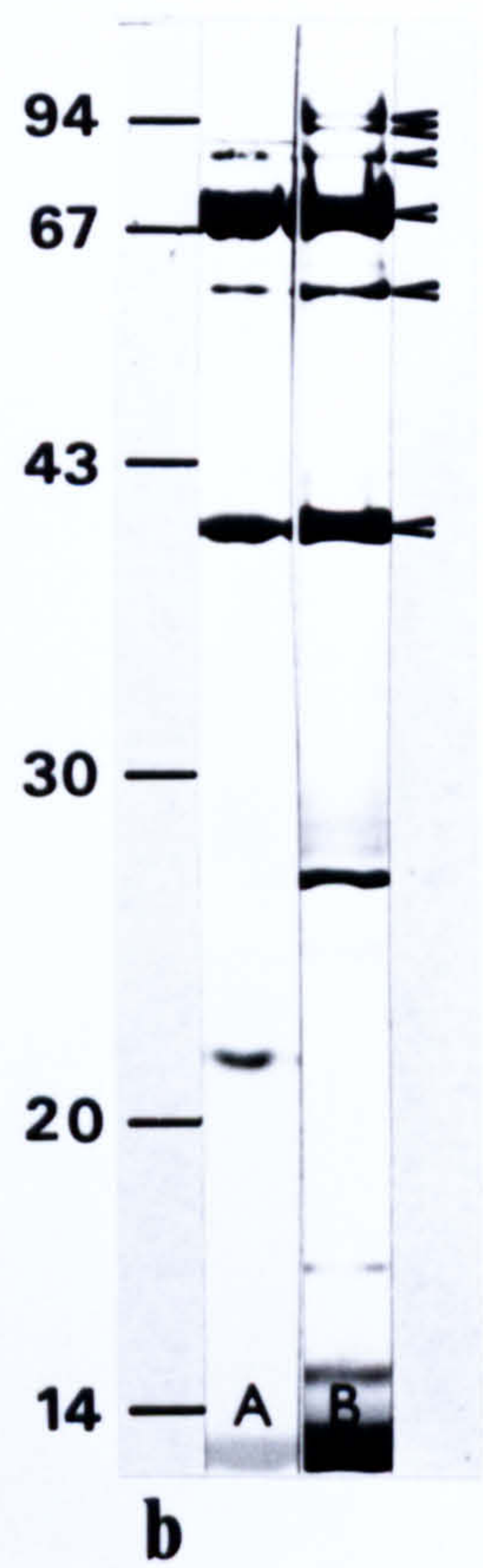


TABLE 3.9 SUMMARY OF MAJOR ANTIGENS IDENTIFIED IN AMMONIUM SULPHATE PARTITIONED FRACTIONS OF RH.
APPENDICULATUS UNFED NYMPHAL EXTRACT.

ANTISERUM	FRACTION	ANTIGEN MOLECULAR WEIGHT (kDa)											
		97.5	93.5	84	77.5	75	70	60	55	51.5	40	36.5	23
Antiadult	20% precipitate	-	-	-	-	-	+/-	-	+/-	-	+++	+++	+++
	35% precipitate	-	-	-	-	-	+/-	-	+/-	-	+++	+++	++
	45% precipitate	-	-	+/-	+/-	-	+/++	-	+/++	-	+	-	-
	45% supernate	-	-	-	-	-	+	-	-	-	+	-	-
Anti45supt	20% precipitate	++	++	+	-	-	+++	++	-	-	++	-	-
	35% precipitate	++	++	+	++	+	+++	++	+	-	++	-	-
	45% precipitate	+++	+++	+++	+++	+++	+++	+++	+	+/-	+/++	-	-
	45% supernate	+/-	+	+/-	-	-	+++	+++	-	-	+++	-	-
Anti45supt low dose	45% supernate	-	-	++	-	-	+++	++	-	-	++	-	-
Anti45supt high dose	45% supernate	+/-	+	+	-	-	+++	++	-	-	+++	-	-
AntiNCP antigens	45% supernate	++	++	+/-	-	-	+++	++	-	-	+	-	-

- Unlabelled +/- Weakly labelled + Clearly labelled ++ Strongly labelled
+++ Intensely labelled UNE Unfed Nymphal Extract NCP Nitrocellulose Paper

3.5.2.3 Ammonium Sulphate Partitioned Fractions of *Rh. appendiculatus* Unfed Nymphal Extract.

UNE fractionated with ammonium sulphate was probed with anti45supt serum and with antiadult *Rh. appendiculatus* serum (figure 3.5.2.3a).

The antiadult serum identified weakly staining bands at 84 and 77.5kDa together with a diffuse band of 70kDa in the 45% ammonium sulphate precipitate; this 70kDa species was also recognised in the 45% supernate as a clearly labelled band. The 70kDa species was observed as a minor weakly labelled component of both the 20% and 35% precipitates. A 55kDa antigen was observed mainly in the 45% precipitate, but was also present to a lesser extent in the 35% and 20% precipitates. A 40kDa antigen was observed in the 45% supernate; this band was also weakly labelled in the 35% and 45% precipitates. Intensely stained bands of 36.5kDa were identified in the 20% and 35% ammonium sulphate precipitates, and staining of the 23kDa species was intense in the 20% precipitate whilst being slightly less so in the 35% precipitate. Neither of these species were identified by antiadult serum in either the 45% precipitate or 45% supernate. The antiserum failed to recognise bands of 97.5, 93.5, 75, 60 and 51.5kDa in any of the fractions.

When probed with anti45supt serum, the 45% precipitate displayed the greatest array of antigens. The 97.5, 93.5, 84, 77.5, 75, 70, and 60kDa species were all very intensely stained, whilst

bands at 55, 51.5 and 40kDa were less strongly so. All of these bands were also identified in the 35% precipitate to a lesser degree, though most were strongly stained, particularly the 70kDa species. The 97.5, 93.5, 84, 70, 60 and 40kDa antigen species were shown to be present in the 20% precipitate; the 70kDa band being the most strongly stained.

A large array of other proteins/polypeptides were labelled to a lesser degree in the 20%, 35% and 45% precipitates; in comparison, the anti45supt serum recognised only a limited number of antigenic bands in the 45% supernate. The 97.5, 93.5 and 84kDa species were all labelled, though not strongly, whilst the 70 and 60kDa bands were intensely stained but not as heavily as in the 45% precipitate. The 40kDa species stained more intensely in the 45% supernate than in any of the other fractions.

Immunisation of guinea pigs with UNE ammonium sulphate 45% supernate at a low dose rate ($135\mu\text{g.guinea pig}^{-1}$) resulted in an antiserum that failed to identify the 97.5kDa or the 93.5kDa bands in the immunising supernate. However, the 70 and 40kDa species were present as strongly stained diffuse bands, and a sharp band of 60kDa was also strongly labelled along with one at 84kDa. When the supernate was probed with antiserum against a higher dose 45% supernate ($1350\mu\text{g.guinea pig}^{-1}$), both the 97.5kDa and 93.5kDa antigens were identified in addition to the 84, 70, 60 and 40kDa bands (figure 3.5.2.3b).

UNE 45% ammonium sulphate supernate was probed with antiserum raised against nitrocellulose antigen-bearing particles (figure 3.5.2.3c). Antigens were identified at 97.5, 93.5, 84, 70, 60 and 40kDa. The 70kDa species was most intense, with the 97.5, 93.5 and 60kDa antigens being strongly labelled.

The locations of major antigens identified in this section are summarised in table 3.9.

3.5.3 Electrophoresis and Western Blotting of Membrane Bound and Soluble Antigens of *Rh. appendiculatus* Unfed Larval Extract.

The antigen profiles of membrane-bound protein and soluble protein fractions of UNE (see 2.3.8), were compared by probing blots with antiF2 and anti45supt sera (figure 3.5.3).

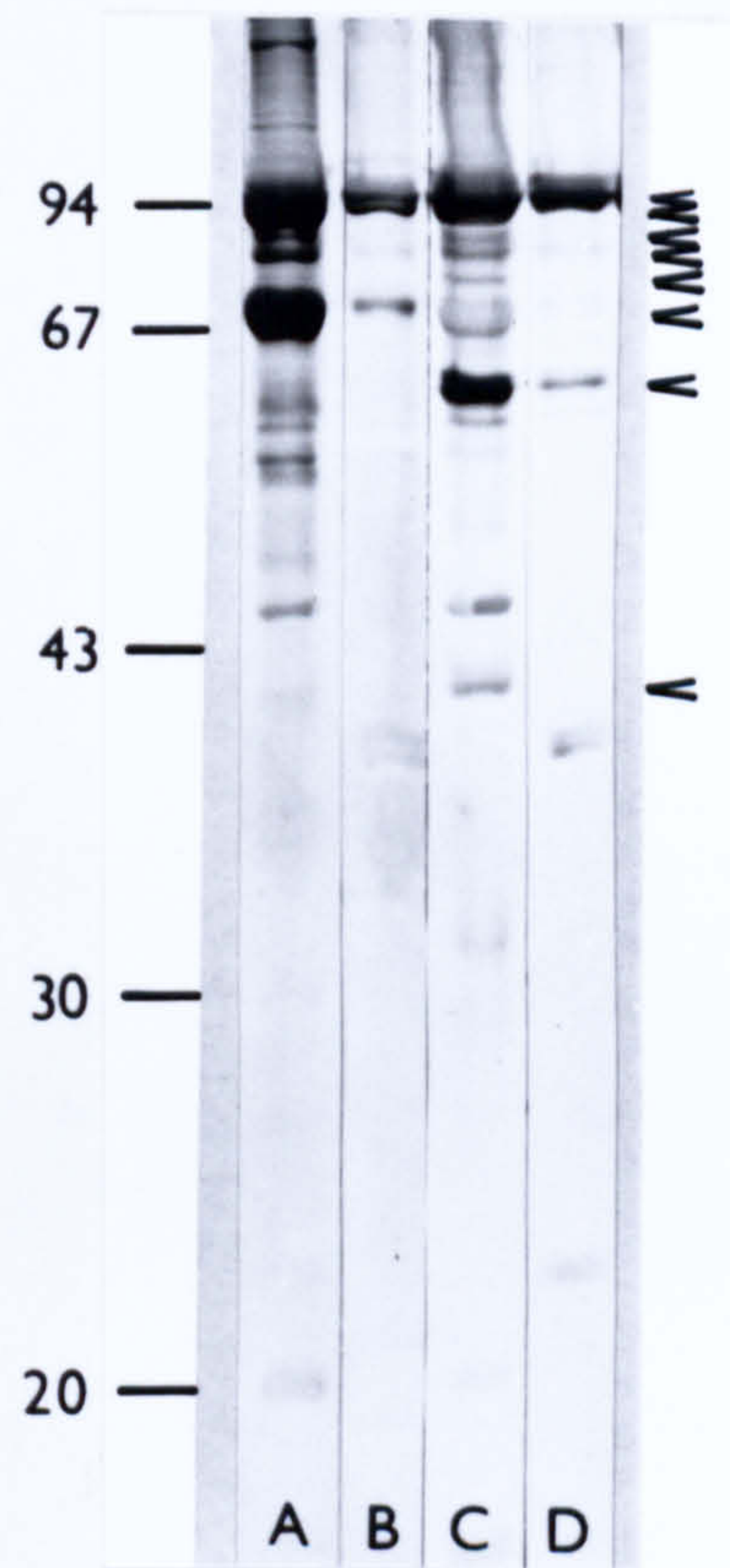
Relatively few antigens, compared to the parent UNE, appeared membrane bound. The 97.5 and 93.5kDa bands were identified in both membrane and soluble fractions by each of the two antisera; a band of 70kDa was also identified in both fractions, but only by the antiF2 serum, not by the anti45supt serum. Bands of 84 and 77.5kDa were identified in the soluble fraction by both antisera. They were not detected in the membrane fraction. A 75kDa antigen was identified only in the soluble fraction and only by the anti45supt serum, and the 60kDa species was identified in both fractions by the anti45supt serum, but not identified by the antiF2 serum. Lastly, a 40kDa antigen was

Figure 3.5.3 Identification of Antigens in *Rh. appendiculatus* Unfed Nymphal Extract Soluble and Membrane Protein Fractions using Guinea Pig Post Immunisation Sera. Lanes A,C unfed nymphal extract (UNE) membrane protein fraction; B,D UNE soluble protein fraction. A,B probed with antiF2 serum; C,D probed with anti45supt serum. Bands referred to in text are arrowed to the right of lane D. Numbers to the left of lane A represent the molecular weights (kDa) of protein standards (listed in Figure 3.5.1.1).

Figure 3.5.4a Identification of Antigens in *Rh. appendiculatus* Unfed Nymphal Extract/ Antilarval Serum (Rabbit and Guinea Pig) Complexes using Rabbit and Guinea Pig Post Infestation (Adult Male and Female) Sera. Lane A unfed nymphal extract/guinea pig antilarval serum complex; B UNE/rabbit antilarval serum complex. A probed with guinea pig antiadult (2 sequential infestations with *Rh. appendiculatus* adults) serum; B probed with rabbit antiadult serum. Bands referred to in text are arrowed to the right of lane B. Numbers to the left of lane A represent the molecular weights (kDa) of protein standards (listed in Figure 3.5.1.1).

Figure 3.5.4b Identification of Antigens in *Rh. appendiculatus* Unfed Nymphal Extract/Guinea Pig Antilarval Serum Complexes using Guinea Pig Post Immunisation Sera. Lanes A and B unfed nymphal extract/guinea pig antilarval serum complex. A probed with anti45supt serum; B probed with anti(UNE/GP antilarval serum complex) serum. Bands referred to in text are arrowed to the right of lane B. Numbers to the left of lane A represent the molecular weights (kDa) of protein standards (listed in Figure 3.5.1.1).

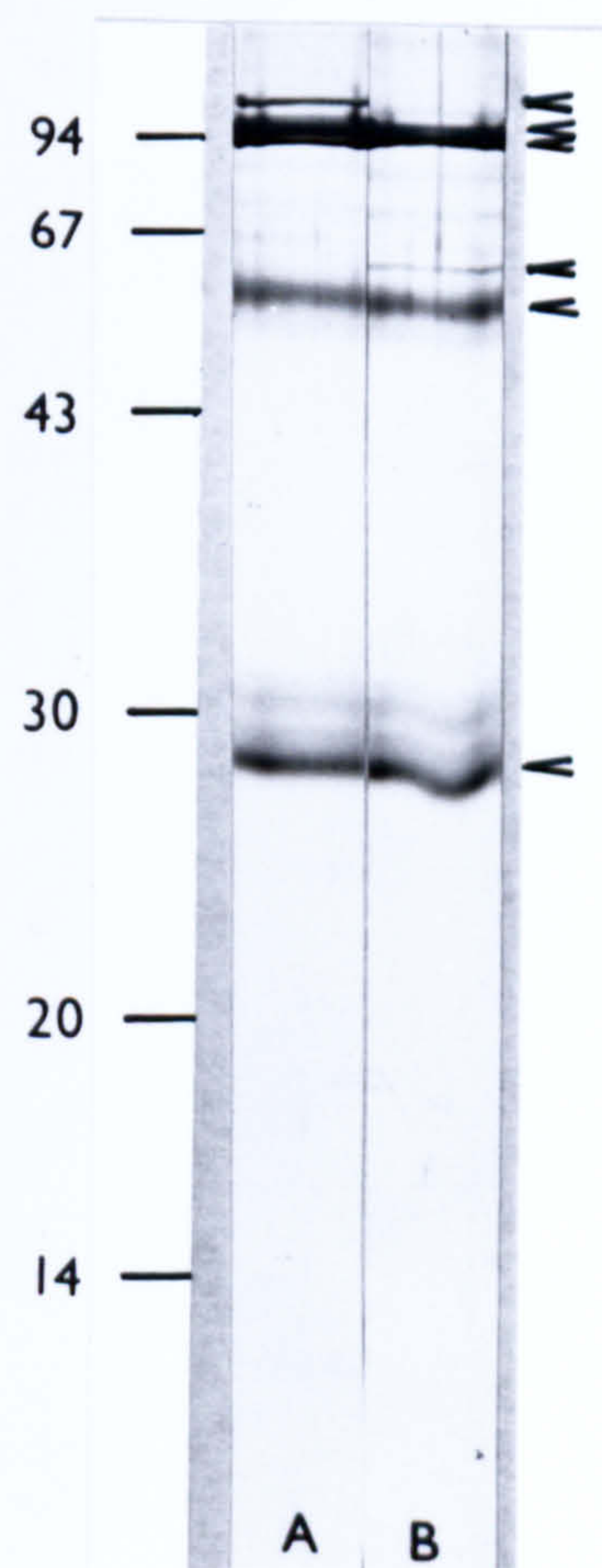
3.5.3



3.5.4



a



b

TABLE 3.10 SUMMARY OF MAJOR ANTIGENS IDENTIFIED IN MEMBRANE BOUND AND SOLUBLE FRACTIONS OF RH. APPENDICULATUS UNFED NYMPHAL EXTRACT.

ANTISERUM	FRACTION	ANTIGEN MOLECULAR WEIGHT (kDa)							
		97.5	93.5	84	77.5	75	70	60	40
AntiF2	Membrane	+ / ++	++	-	-	-	+	-	-
	Soluble	+++	+++	++	++	-	+++	-	-
Anti45supt	Membrane	++	++	-	-	-	-	+	-
	Soluble	++	++	+	+	+	+	++	+

- Unlabelled +/- Weakly labelled + Clearly labelled ++ Strongly labelled
+++ Intensely labelled UNE Unfed Nymphal Extract

TABLE 3.11 SUMMARY OF MAJOR ANTIGENS IDENTIFIED IN IMMUNE COMPLEXES (RH. APPENDICULATUS UNFED NYMPHAL EXTRACT WITH RABBIT OR GUINEA PIG ANTILARVAL RH. APPENDICULATUS SERA).

ANTISERUM	UNE COMPLEXED WITH SERUM FROM	ANTIGEN MOLECULAR WEIGHT (kDa)											
		140	97.5	93.5	84	77.5	60	55	51.5	36.5	27	23	
Antiadult	Guinea Pig	-	-	-	-	-	-	-	++	++	+	+++	
	Rabbit	-	-	-	+	+	-	-	++	++	-	+++	
Antiguinea pig complex	Guinea Pig	++	+++	+++	-	-	-	+ / ++	-	-	+ / ++	-	
Anti45supt	Guinea Pig	-	++	++	-	-	+	+ / ++	-	-	+ / ++	-	

- Unlabelled +/- Weakly labelled + Clearly labelled ++ Strongly labelled
 +++ Intensely labelled UNE Unfed Nymphal Extract

identified by the anti45supt serum in the soluble protein fraction. This species was not identified in the membrane fraction (table 3.10).

3.5.4 Electrophoresis and Western Blotting of *Rh. appendiculatus* Immune Complexes.

Immune complexes (see 2.3.7) were probed with rabbit or guinea pig antiadult serum (2 sequential infestations with *Rh. appendiculatus* adults) (figure 3.5.4a). When the UNE-guinea pig antiserum complex (2 sequential infestations with *Rh. appendiculatus* larvae) was probed with guinea pig antiadult serum, strong bands were observed at 51.5, 36.5 and 23kDa, the latter two being particularly intense. An additional diffuse band at 27kDa was also observed. The UNE-rabbit antiserum complex (2 sequential infestations with *Rh. appendiculatus* larvae), when probed with rabbit antiadult serum, exhibited an intense band at 23kDa, and strong banding at 51.5 and 36.5kDa. Two further antigenic species were observed at 84 and 77.5kDa.

When the UNE-guinea pig antiserum complex was probed with its own antiserum intense bands were observed at 97.5 and 93.5kDa, together with a strong band at *ca.* 140kDa and diffuse bands at 55 and 27kDa. No species at 36.5 or 23kDa were detected. On probing the complex with anti45supt serum bands, at 97.5, 93.5, 55 and 27kDa were seen, but not the 140kDa species; an additional band at 60kDa was clearly observed (figure 3.5.4b).

The locations of major antigens identified in this section are summarised in table 3.11.

3.5.5 Electrophoresis and Western Blotting of Whole Tick Extracts from Different Species.

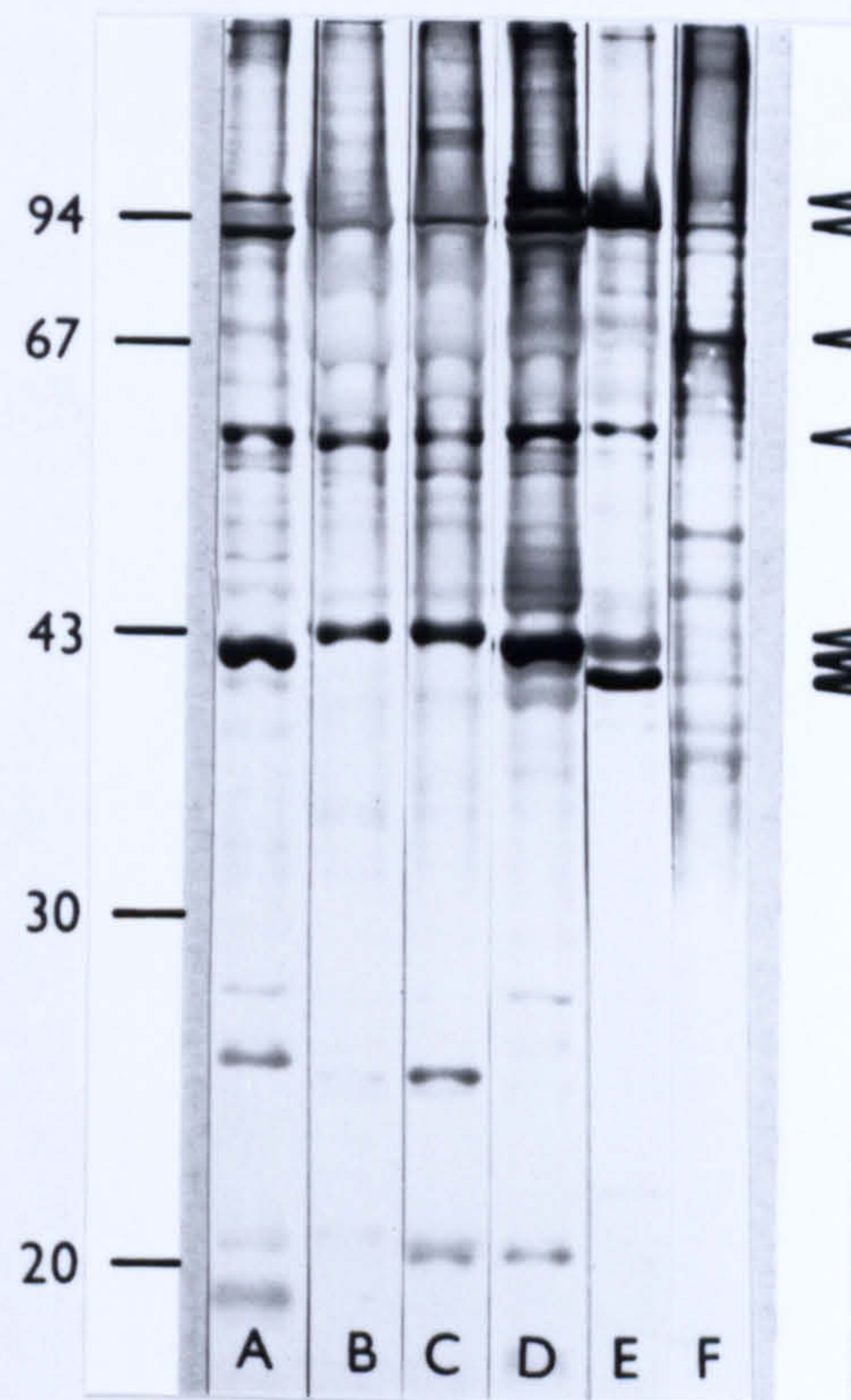
In an attempt to identify cross-reacting (core/common) antigens capable of inducing immunity to more than one species (figure 3.5.5) unfed larval extracts of *Am. hebraeum*, *Am. variegatum*, *Bo. microplus*, *Rh. appendiculatus* and *Rh. evertsi evertsi* were probed with anti45supt serum. The antiserum labelled antigens of ca. 100kDa in *Bo. microplus* and *Rh. e. evertsi* ULEs, and 93.5kDa in *Bo. microplus*, *Rh. appendiculatus* and *Rh. e. evertsi* extracts. Antigenic species of 60kDa were identified in all five of the extracts, but a band at 44kDa was labelled only in the *Am. hebraeum* and *Am. variegatum* ULEs. The antiserum also located 42.5kDa species in ULEs of *Bo. microplus*, *Rh. appendiculatus* (can be identified in figure 3.5.1.2, though not referred to in text) and *Rh. e. evertsi*. In the *Bo. microplus* ULE a band at 40kDa was weakly labelled, whilst a 40kDa species in the *Rh. appendiculatus* extract was more strongly identified (table 3.12).

A mixed adult extract of *Brugia pahangi*, a parasitic helminth, was probed with the same antiserum as above. A wide variety of polypeptide bands were weakly labelled, whilst a more strongly stained band was observed at 70kDa.

Figure 3.5.5 Identification of Antigens in Unfed Larval Extracts of Different Tick Species and an Extract of *Brugia pahangi* (a Parasitic Helminth) Mixed Adults using Post Immunisation (*Rh. appendiculatus*) Serum. Lane A *Bo. microplus* unfed larval extract (ULE); B *Am. hebraeum* ULE; C *Am. variegatum* ULE; D *Rh. e. evertsi* ULE; E *Rh. appendiculatus* ULE; F *Brugia pahangi* mixed adult extract. All extracts were probed with *Rh. appendiculatus* anti45supt serum (UNE fraction). Bands referred to in text are arrowed to the right of lane F. Numbers to the left of lane A represent the molecular weights (kDa) of protein standards (listed in Figure 3.5.1.1).

Figure 3.5.6 Identification of Glycosylated Proteins/Polypeptides in *Rh. appendiculatus* Unfed Nymphal Extract and Its 45% Ammonium Sulphate Supernate Fraction using Peroxidase-Conjugated Lectins. A,C,E,G,I,K unfed nymphal extract (UNE); B,D,F,H,J,L UNE 45% ammonium sulphate supernate fraction. A,B probed with aparagus pea lectin; C,D probed with winged bean lectin; E,F probed with wheat germ agglutinin; G,H probed with concanavalin A; I,J probed with peanut agglutinin; J,K probed with the lectin BS-1. Bands referred to in text are arrowed to the right of lane L. Numbers to the left of lane A represent the molecular weights (kDa) of protein standards (listed in Figure 3.5.1.1).

3.5.5



3.5.6

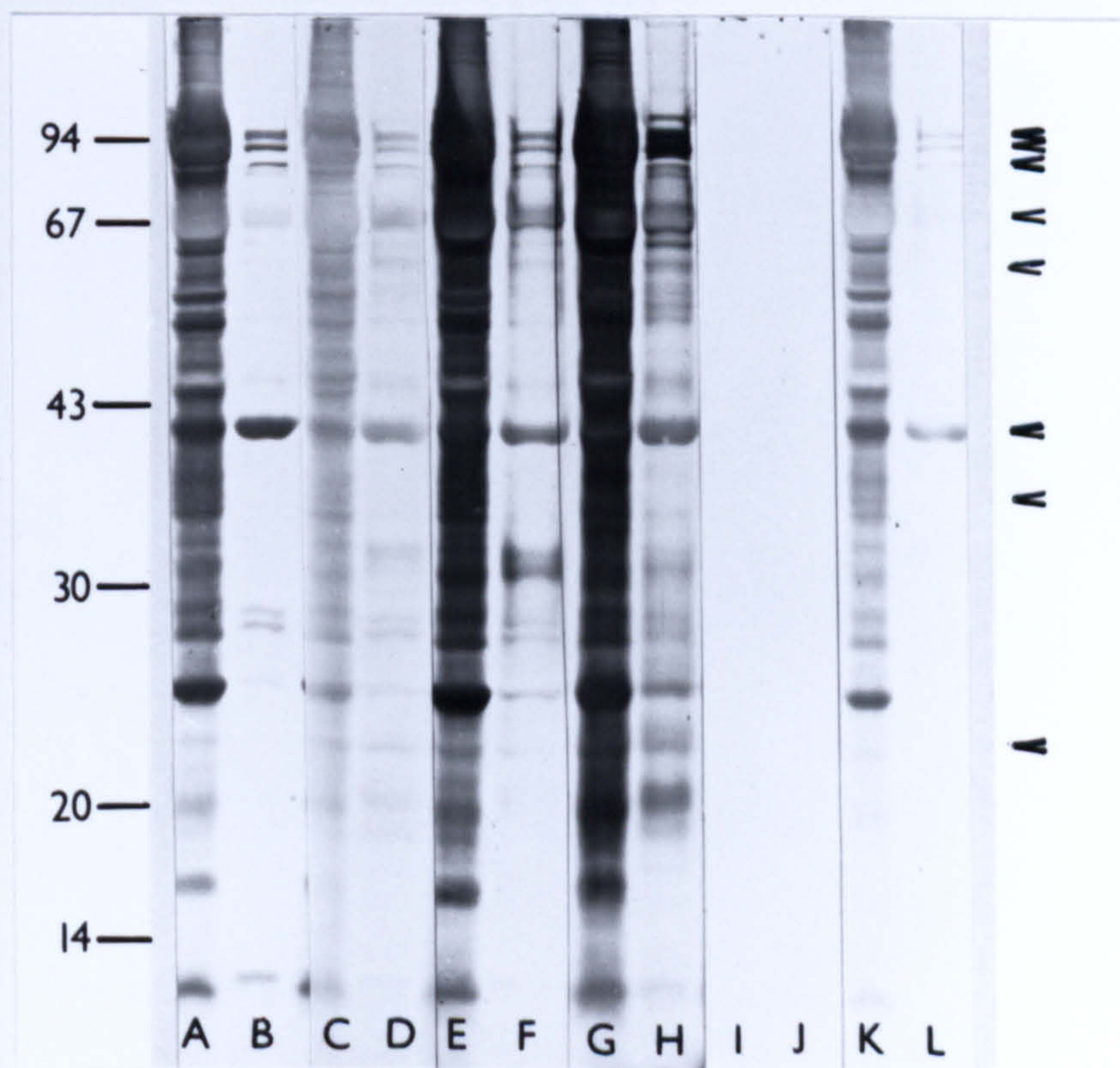


TABLE 3.12 SUMMARY OF CROSS-REACTING ANTIGENS FROM UNFED LARVAL EXTRACT'S OF DIFFERENT SPECIES IDENTIFIED BY PROBING EXTRACTS WITH GUINEA PIG ANTI45SUPT (RH. APPENDICULATUS UNE) SERUM.

SPECIES	ANTIGEN MOLECULAR WEIGHT (kDa)						
	100	93.5	70	60	44	42.5	40
<i>An. hebraeus</i>	-	-	-	+ / ++	++	-	-
<i>An. variegatus</i>	-	-	-	+ / ++	++	-	-
<i>Bo. microplus</i>	+	+	-	+ / ++	-	++	+ / -
<i>Rh. appendiculatus</i>	-	+	-	+ / ++	-	+	++
<i>Rh. e. evertsi</i>	++	+	-	++	-	++	-
<i>Brugia pahangi</i> mixed adult extract	+ / -	+ / -	+ / ++	+ / -	+ / -	+ / -	+ / -

- Unlabelled + / - Weakly labelled + Clearly labelled
 ++ Strongly labelled +++ Intensely labelled
 ULE Unfed Larval Extract UNE Unfed Nymphal Extract

TABLE 3.13 PROPERTIES OF LECTINS USED IN EXPERIMENT 3.5.6.

COMMON NAME	SOURCE	CARBOHYDRATE SPECIFICITY*	METAL ION REQUIREMENT
Peanut agglutinin; PA	<i>Arachis hypogaea</i> (peanut)	β -D-Galactose	Zn^{2+} Ca^{2+}
BS-1	<i>Bandeiraea simplicifolia</i>	α -D-Galactose N-Acetyl- -D-Galactose	Mn^{2+} Ca^{2+}
Concanavalin A; Con A	<i>Canavalia ensiformis</i> (jack bean	α -D-Mannose α -D-Glucosamine N-Acetyl- α -D-Glucosamine	Mn^{2+} Ca^{2+}
Winged bean	<i>Psophocarpus tetragonolobus</i>	N-Acetyl-D-Galactosamine	Mn^{2+} Ca^{2+}
Winged Pea; Asparagus Pea	<i>Tetragonolobus purpureas</i>	α -L-Fucose	Zn^{2+} Ca^{2+}
Wheat germ agglutinin; WGA	<i>Triticum vulgaris</i> (wheat germ)	N-Acetyl-D-Glucosamine N-Acetyl-Neuraminic Acid	Mn^{2+} Zn^{2+} Ca^{2+}

* Where greater than one group of carbohydrates is listed, these are given in decreasing order of affinity Ca^{2+} Calcium ions Mn^{2+} Manganese ions Zn^{2+} Zinc ions

TABLE 3.14 CARBOHYDRATE MOIETIES OF MAJOR ANTIGENS IDENTIFIED IN RH. APPENDICULATUS UNFED NYMPHAL EXTRACT.

LECTIN	CARBOHYDRATE MOIETIES	ANTIGEN MOLECULAR WEIGHT (kDa)							
		97.5	93.5	84	70	60	40	36.5	23
Peanut agglutinin	-D-Galactose	-	-	-	-	-	-	-	-
BS-1	-D-Galactose	+/-	+/-	+/-	+/-	-	+	-	-
	N-Acetyl -D-Galactose								
Con A	-D-Mannose	+++	+++	++	++	+/-	++	++	++
	-D-Glucosamine								
	N-Acetyl- -D-Glucosamine								
Winged Bean	N-Acetyl-D-Galactosamine	+/-	+/-	+/-	+/-	+/-	++	+/-	+/-
Asparagus pea	-L-Fucose	++	++	++	+/+	+/-	+++	+/-	+/-
Wheat Germ agglutinin	N-Acetyl-D-glucosamine	++	++	+	++	+/-	++/	++	+/++
	N-Acetyl-Neuraminic Acid						+++		

- Unlabelled +/- Weakly labelled + Clearly labelled ++ Strongly labelled
+++ Intensely labelled BS-1 *Bandeiraea simplicifolia* Con A Concanavalin A

3.5.6 Identification of Carbohydrate Moieties on *Rh. appendiculatus* Tick Antigens using Lectin-Binding Assays.

Rh. appendiculatus UNE and its 45% ammonium sulphate supernate were transferred to nitrocellulose paper after electrophoresis and then processed as in 2.10. Six different horseradish-peroxidase conjugated lectins were used to assay the samples for the presence of particular carbohydrate groups (table 3.13).

The majority of proteins appeared heavily glycosylated, several exhibiting a broad range of carbohydrate moieties (figure 3.5.6). Concanavalin A (predominantly alpha-D-Mannose binding) bound to the majority of protein/polypeptide species in the extracts including those at 97.5, 93.5, 84, 70, 40, 36.5 and 23kDa. The 60kDa band was only weakly bound by con A (table 3.14). Fewer species were identified by wheat germ agglutinin (mainly N-Acetyl-D-Glucosamine binding) than by con A, though all the major antigen species listed above were bound; the 60kDa band was again only very weakly bound. Binding of asparagus pea lectin (alpha-L-Fucose binding) was achieved to some degree by all eight of the major antigen bands. The 40kDa species was particularly intensely bound, and the 70, 60, 36.5 and 23kDa antigens were only very weakly bound. Only the 40kDa species showed clear binding to winged bean lectin (N-Acetyl-D-Galactosamine binding), whilst those at 97.5, 93.5, 84 and 70kDa were only weakly bound and antigens at 36.5 and 23kDa were very weakly bound. The lectin BS-1 (mainly alpha-D-Galactose binding) bound to the 40kDa

band, weakly to the 97.5, 93.5 and 84kDa bands and only very weakly to the 70kDa species. The 60, 36.5 and 23kDa species exhibited no visible indications of binding BS-1. None of the proteins/polypeptides showed any sign of binding to peanut agglutinin (beta-D-Galactose specific).

None of the samples showed any signs of binding to lectins that had been previously incubated with an appropriately specific carbohydrate (see table 3.13), suggesting that non-specific binding of lectin to extract material was minimal.

3.5.7 Immunostaining of Sections of Unfed Female *Rh. appendiculatus* for Antigen Localisation.

Sagittal sections of unfed female ticks were probed with antiadult guinea pig serum or with sera from guinea pigs immunised with UNE or its fractions. Sections probed with normal (naive) guinea pig serum were used as controls. A summary of the results is given in table 3.15.

Tissues in sections probed with normal guinea pig serum (figures 3.5.7a and 3.5.7b) did not stain.

When sections were probed with serum from a highly resistant guinea pig (3 sequential infestations with *Rh. appendiculatus* adults), all three types of salivary gland acini (types I, II and III) were strongly labelled with antibody as were the salivary

ducts and the lumen of the malpighian tubules. The haemolymph and haemocytes showed a low degree of labelling, whilst the synganglion stained to an intensity intermediate to that of the salivary gland acini and the haemolymph. Gut and muscle tissues were unlabelled. Additionally the oesophageal tract was strongly labelled; this was not labelled in any of the other sections (figures 3.5.7c - 3.5.7e).

When sections were probed with serum raised against whole unfed nymphal extract (UNE) virtually all the tissues stained to some degree. The haemocytes were most strongly labelled followed by the synganglion and the haemolymph. Although not illustrated, the type I salivary gland acini were also intensely stained as was the lumen of the malpighian tubules. The lumen of types II and III salivary gland acini were also clearly labelled, and both gut and muscle were weakly stained. The salivary ducts were unstained (figures 3.5.7f and 3.5.7g).

Serum raised against UNE 45% ammonium sulphate supernate labelled strongly type I salivary gland acini, but the type II acini were labelled only in the lumen, and the type III acini appeared unlabelled. The salivary ducts were unlabelled as were the malpighian tubules, muscle and gut tissues. The synganglion showed a very low level of staining, the haemolymph was clearly stained and the haemocytes very strongly stained (figures 3.5.7h - 3.5.7j).

In sections probed with serum raised against the reduced dose UNE 45% ammonium sulphate supernate the haemolymph was clearly stained as were to a lesser extent, the haemocytes, synganglion, muscle tissue and salivary gland type I acini. Types II and III acini, the gut, salivary ducts and malpighian tubules were unstained (figures 3.5.7k - 3.5.7m).

Serum raised against UNE SEHPLC fraction 2 labelled the haemocytes strongly. Salivary gland type I acini (not illustrated) were also intensely stained, but both types II and III acini were unlabelled, as were the gut, salivary ducts, malpighian tubules and muscle tissue. The haemolymph and synganglion were both positively stained (figures 3.5.7n and 3.5.7o).

When sections were probed with antiserum to nitrocellulose antigen-bearing particles the haemolymph was very intensely stained. Both the synganglion and the type I salivary gland acini were also positively labelled; all other tissues, including the haemocytes, were unstained (figures 3.5.7p and 3.5.7q).

(*n.b.* in figures 3.5.7n - 3.5.7q antisera were applied at a dilution of 1:500, in all other cases a concentration of 1:250 was used.)

TABLE 3.15 ANTIGEN LOCALISATION IN SECTIONS OF UNFED FEMALE RH. APPENDICULATUS.

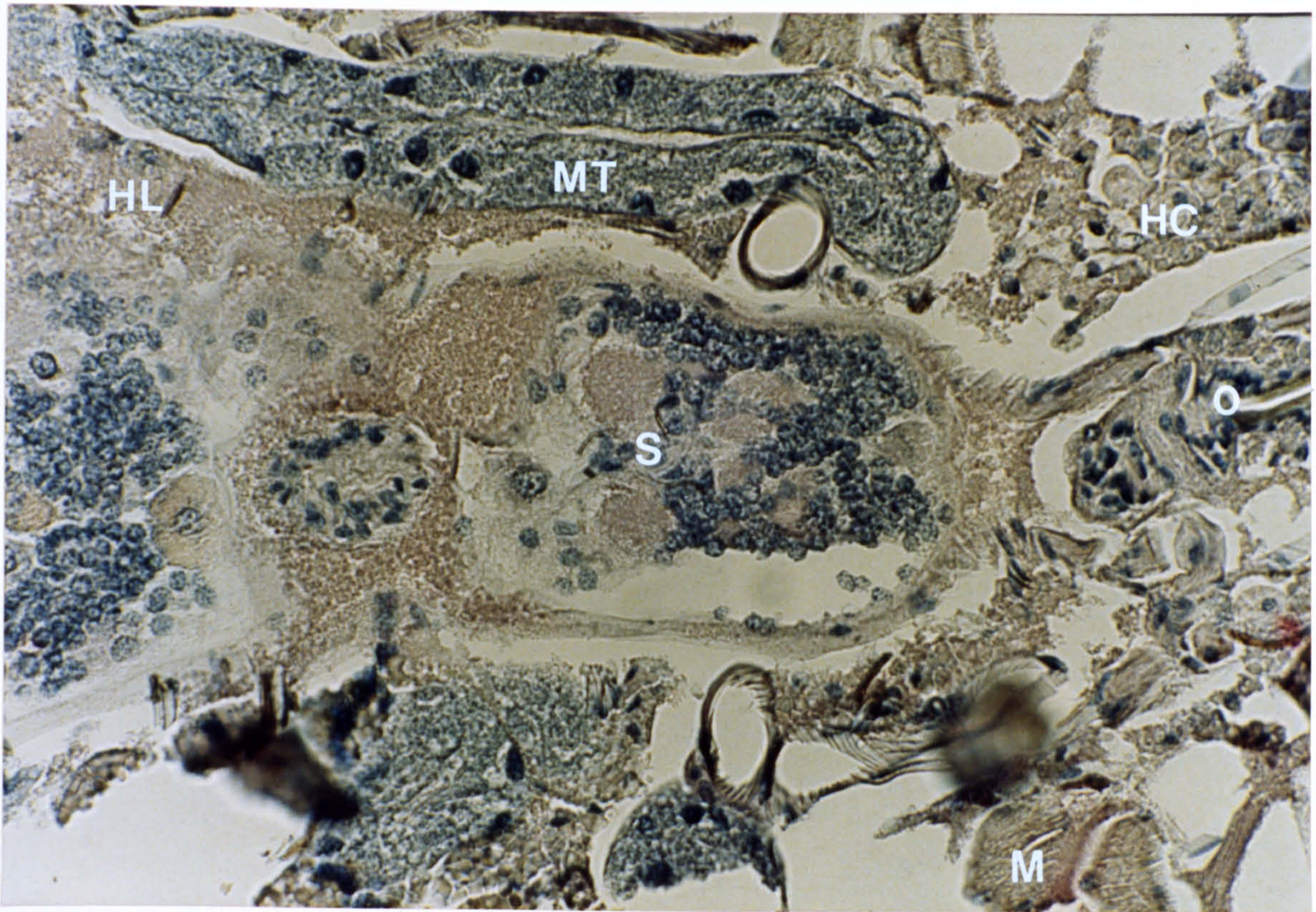
SERUM	TISSUE TYPE																	
	SALIVARY GLAND ACINI TYPE			SALIVARY DUCTS	GUT	MALPHIGIAN TUBULES	MUSCLE	SYN-GANGLION	HAEMO-LYMPH	HAEMO-CYTES	OESO-PHAGUS							
	I	II	III															
NGPS	-	-	-	-	-	-	-	-	-	-	-							
Antiadult	++	++	++	++		++	-	+	+/-	+/-	+++							
		(+++)	(+++)			Lumen only												
AntiUNE	++	++	++	++	+/-	++	+/-	++	++	+++	-							
		Lumen only	Lumen only			Lumen only												
Anti45supt	++	Lumen only	-	-	-	-	-	+/-	+	+++	-							
		Lumen only																
Anti45supt (low dose)	+	-	-	-	-	-	+	+	++	+	-							
AntiF2	++	-	-	-	-	-	-	+	+	++	-							
AntiNCP	+	-	-	-	-	-	-	+	+++	-	-							

- Unlabelled +/- Weakly labelled + Clearly labelled ++ Strongly labelled +++ Intensely labelled
 NGPS Normal guinea pig serum Antiadult 3 sequential infestations with *Rh. appendiculatus* adults
 UNE Unfed Nymphal Extract NCP Nitrocellulose antigen-bearing particles

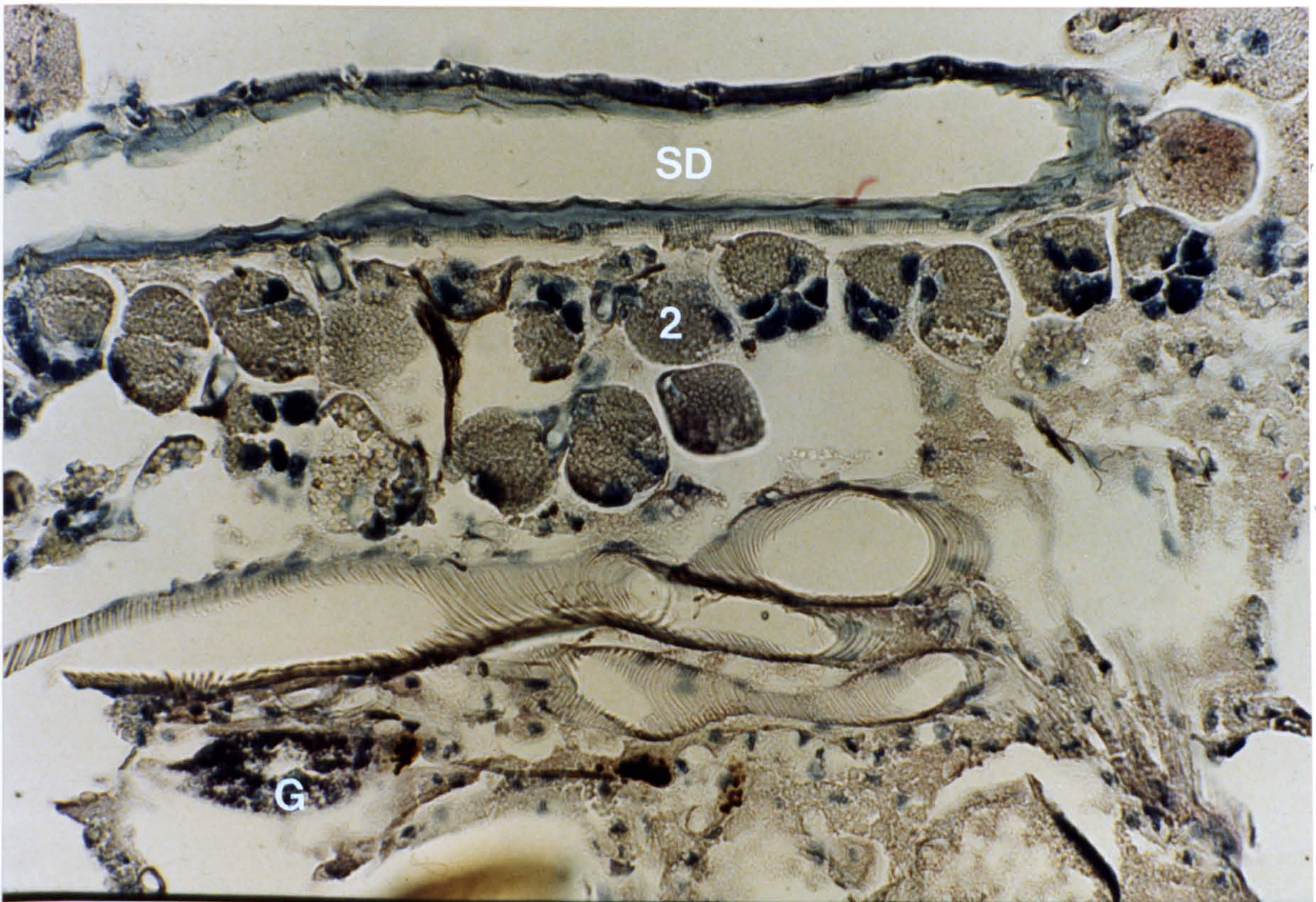
Figure 3.5.7 Antigen Localisation in Sections of Unfed Female *Rh. appendiculatus* Ticks using Post Infestation (Adult Males and Females) and Post Immunisation Sera. Key: G gut; HC haemocytes; HL haemolymph; MT malpighian tubules; M muscle; O oesophagus; SD salivary ducts; 1,2,3 salivary gland acini types I, II and III; S synganglion.

a,b: Tissues Stained using Normal Guinea Pig Serum (Magnification x100).

3.5.7

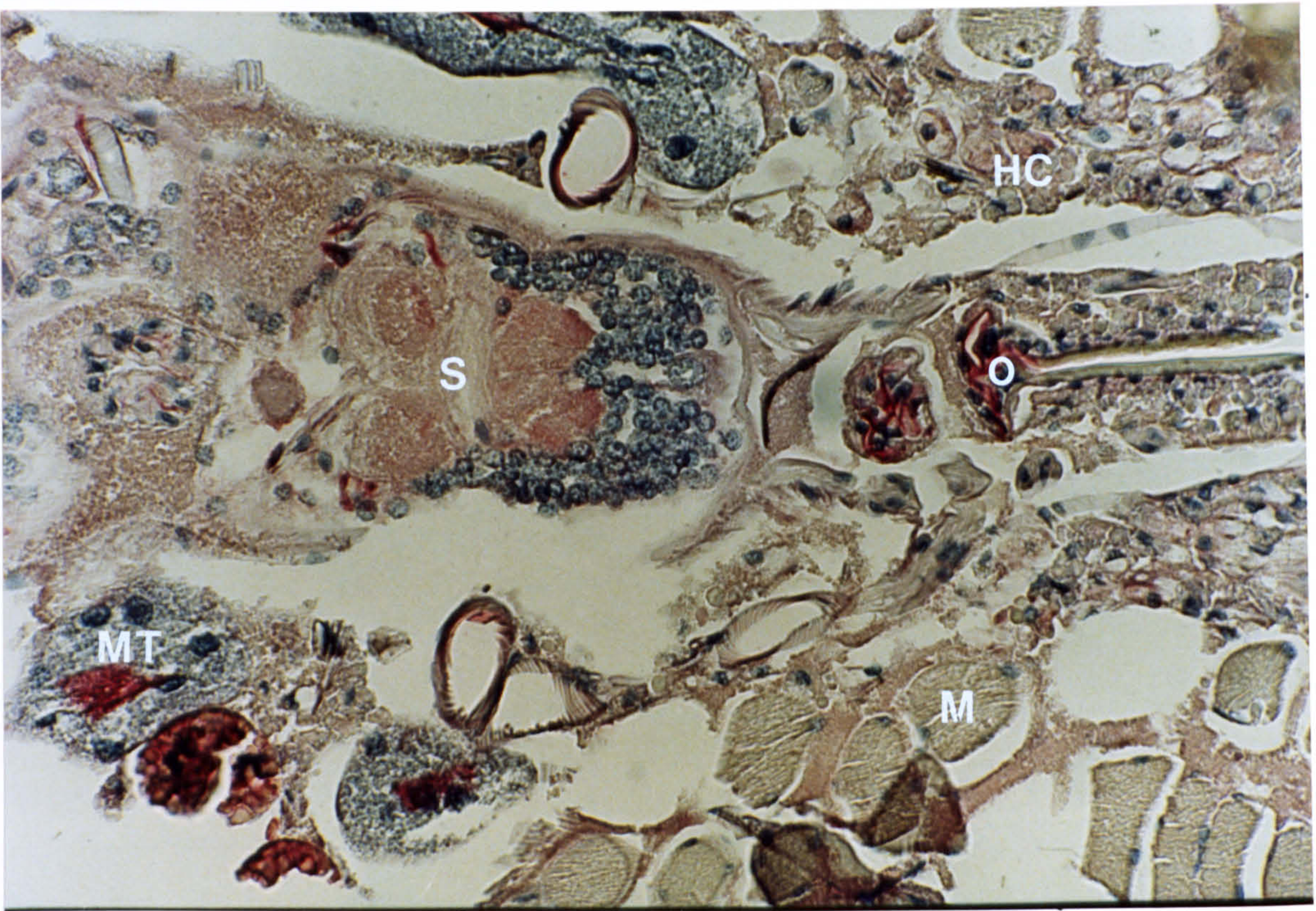


a

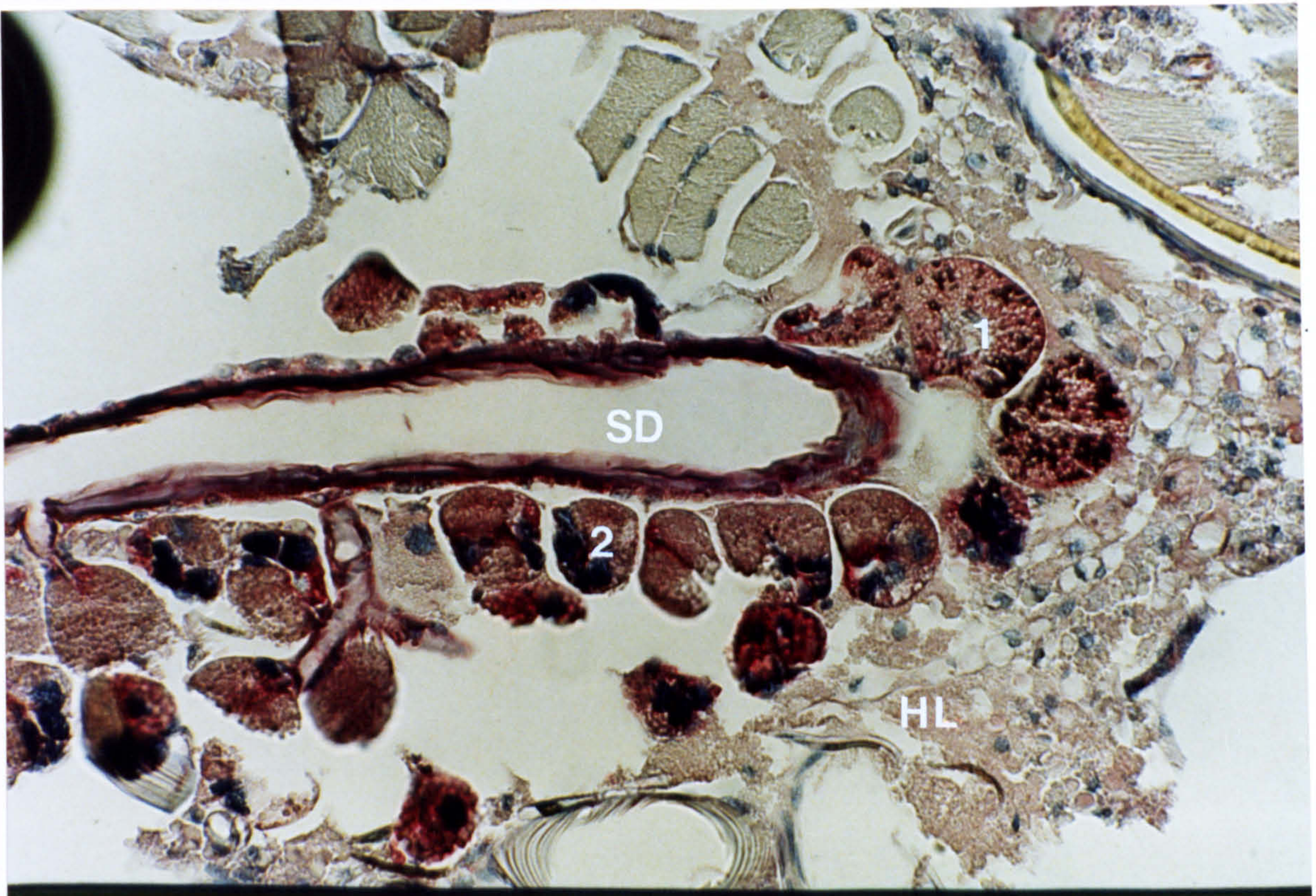


b

c,d: Tissues Stained using Guinea Pig Antiadult (3 Sequential Infestations with *Rh. appendiculatus* Adults) Serum (Magnification x100).



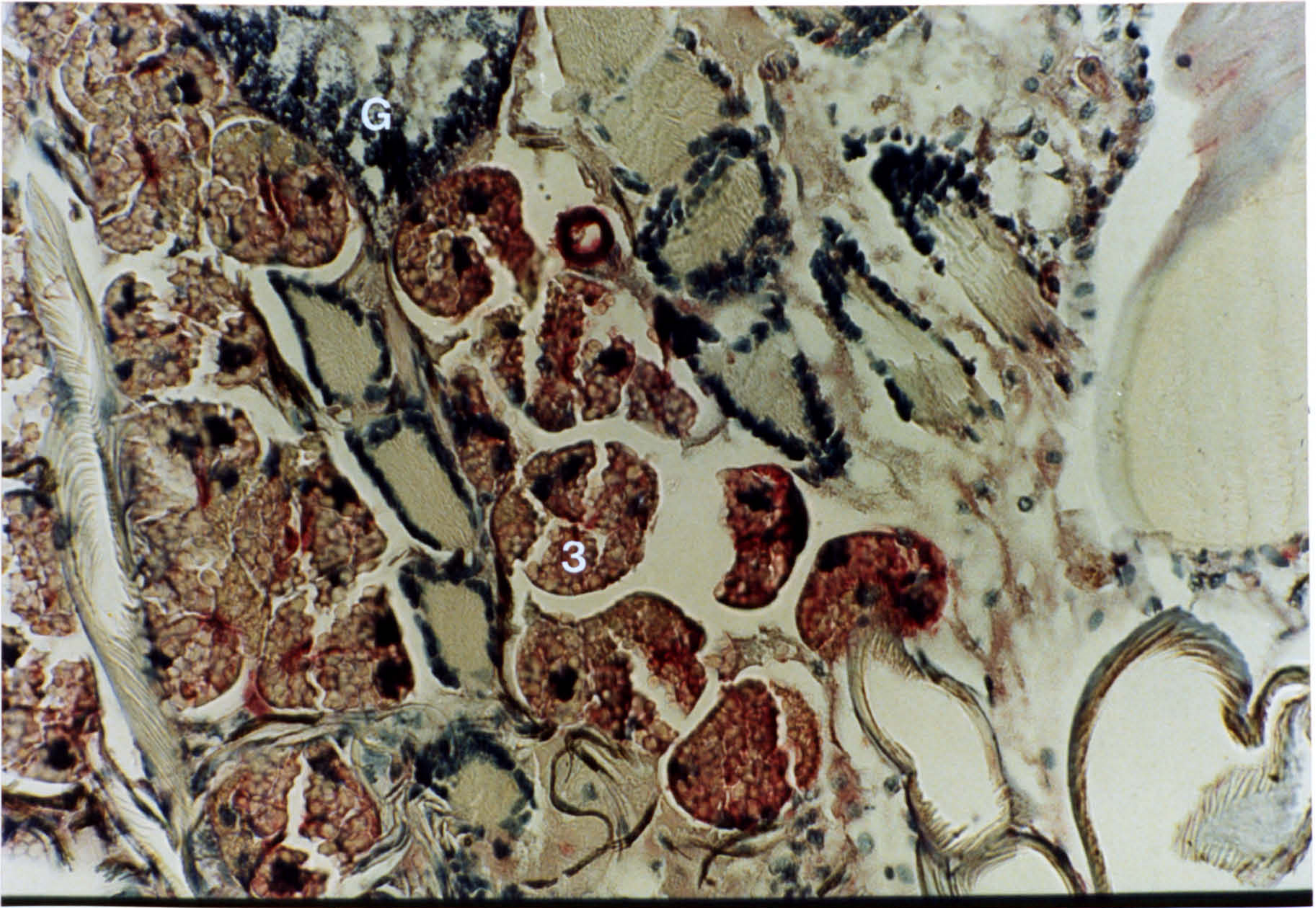
c



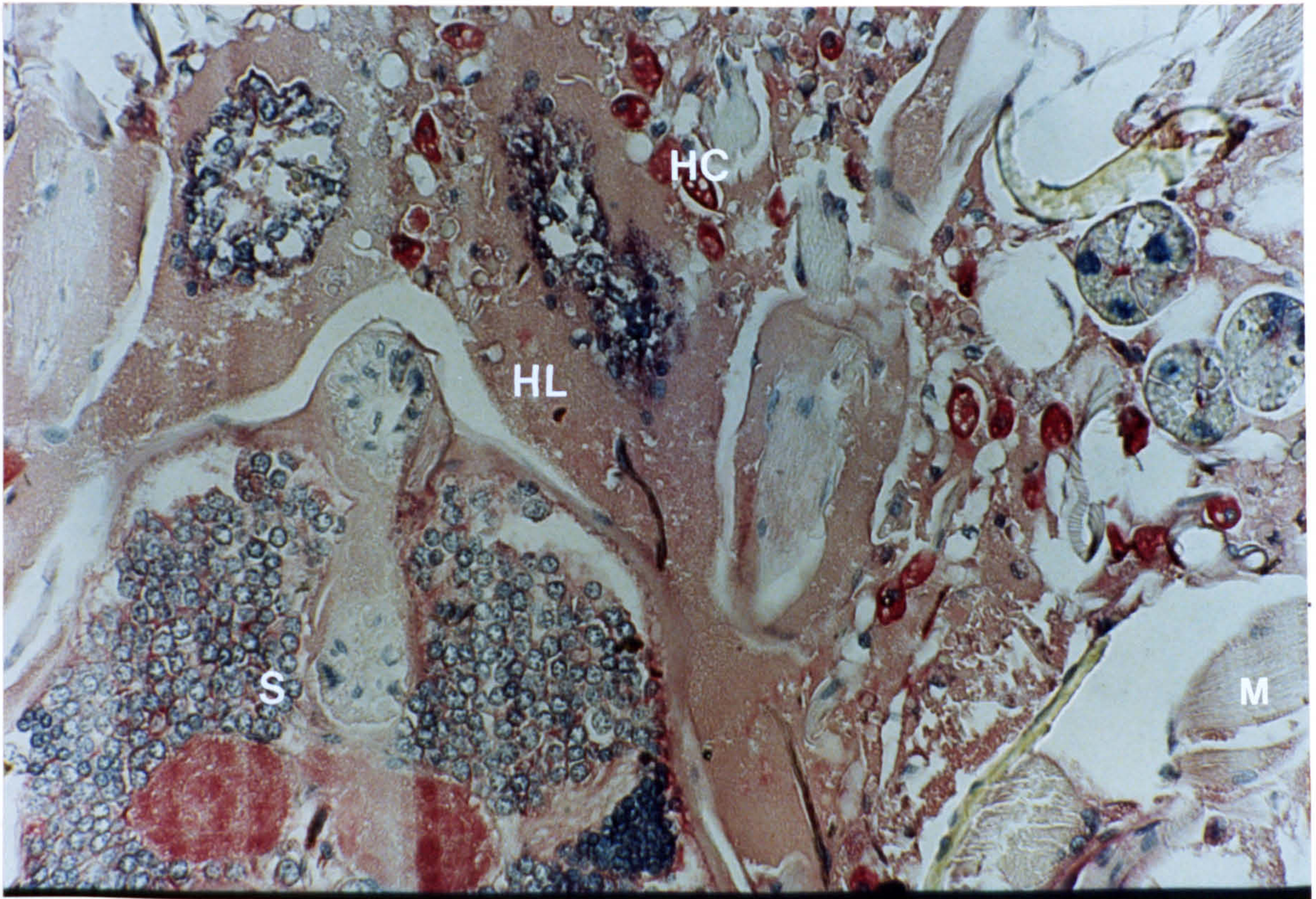
d

e: Tissues Stained using Guinea Pig Antiadult (3 Sequential Infestations with *Rh. appendiculatus* Adults) Serum (Magnification x120).

f: Tissues Stained using Guinea Pig Antiserum against *Rh. appendiculatus* Unfed Nymphal Extract (Magnification x100).



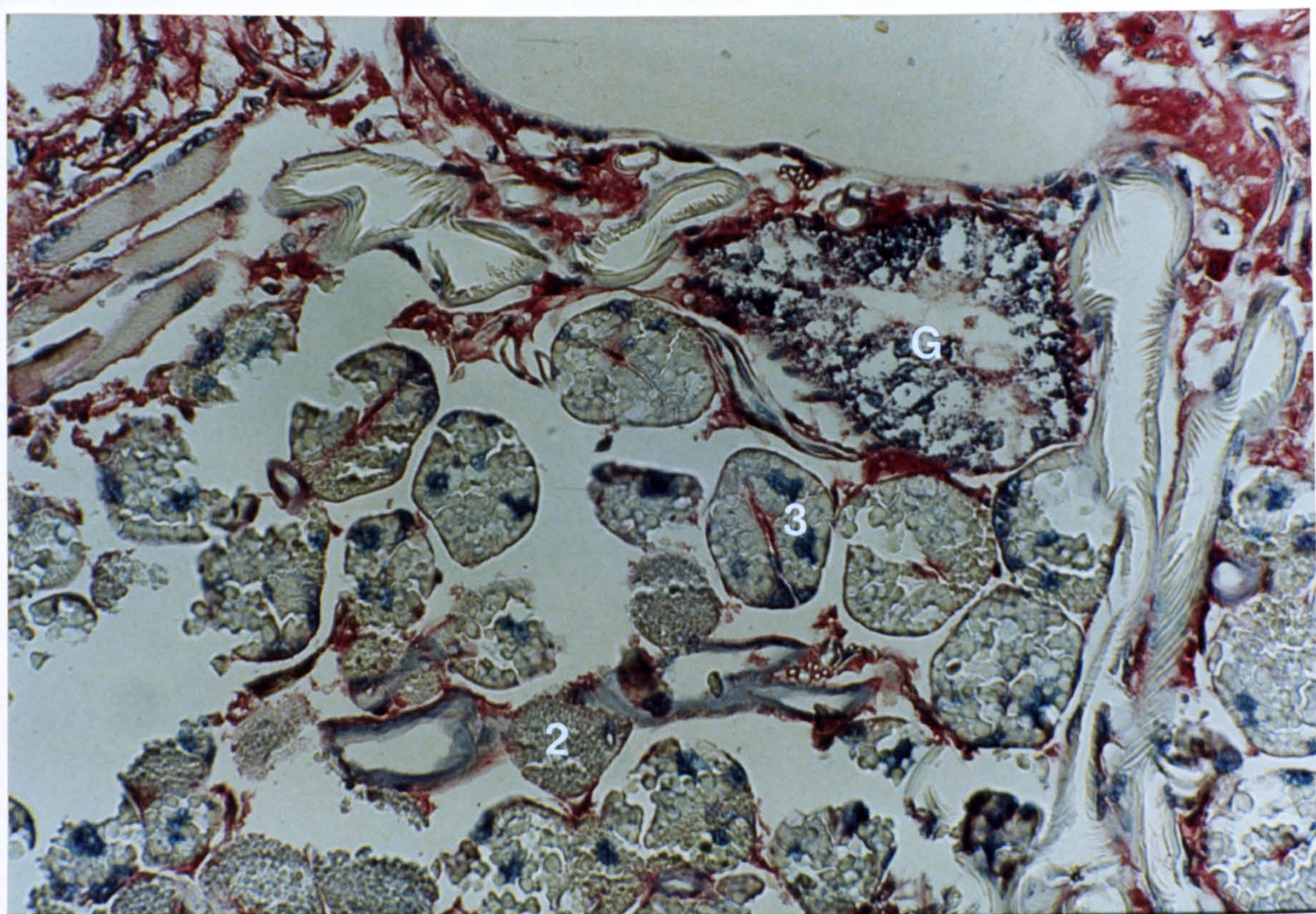
e



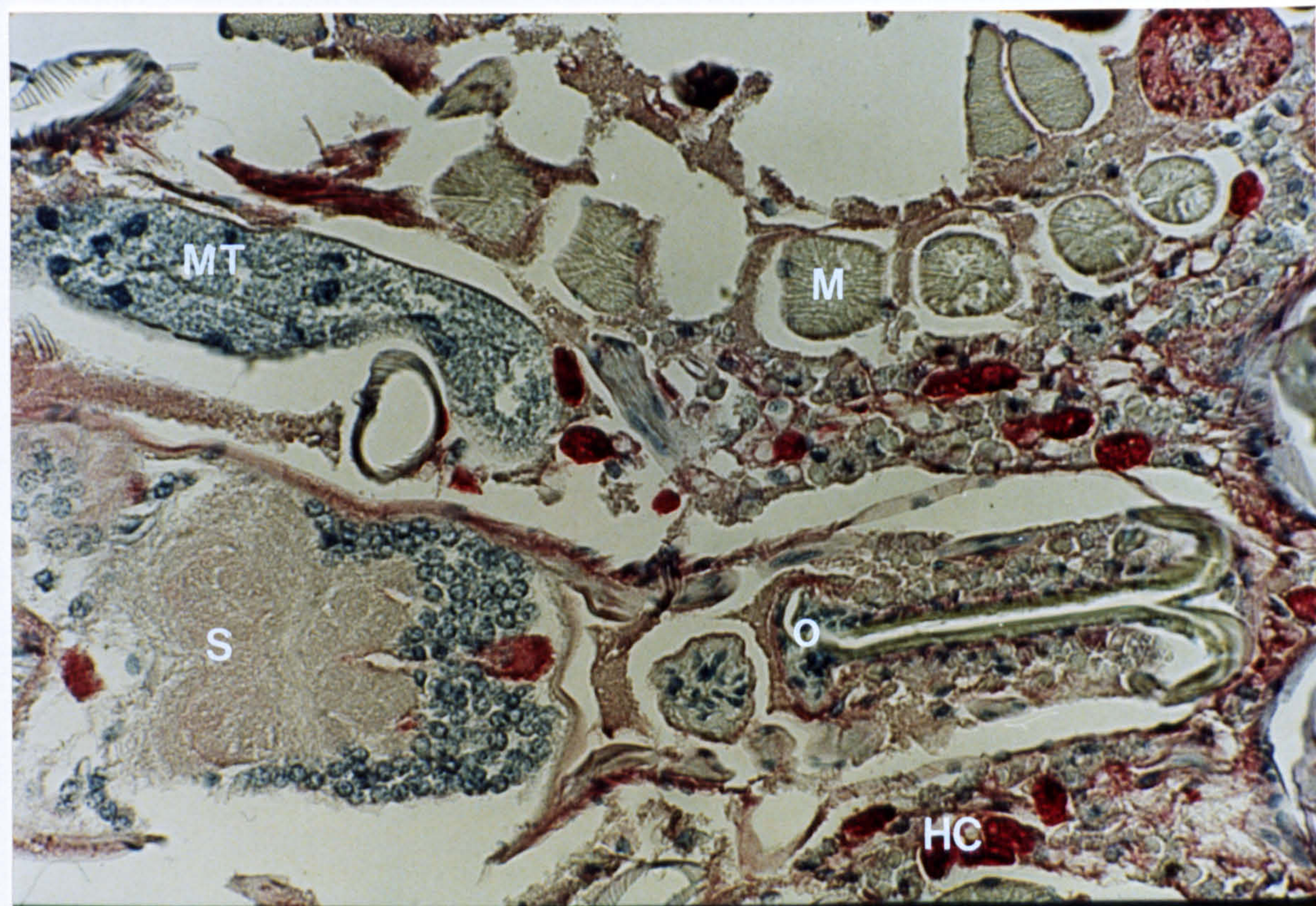
f

g: Tissues Stained using Guinea Pig Antiserum against *Rh. appendiculatus* Unfed Nymphal Extract (Magnification x100).

h: Tissues Stained using Guinea Pig Antiserum against *Rh. appendiculatus* Unfed Nymphal Extract 45% Ammonium Sulphate Supernate Fraction (Magnification x100).

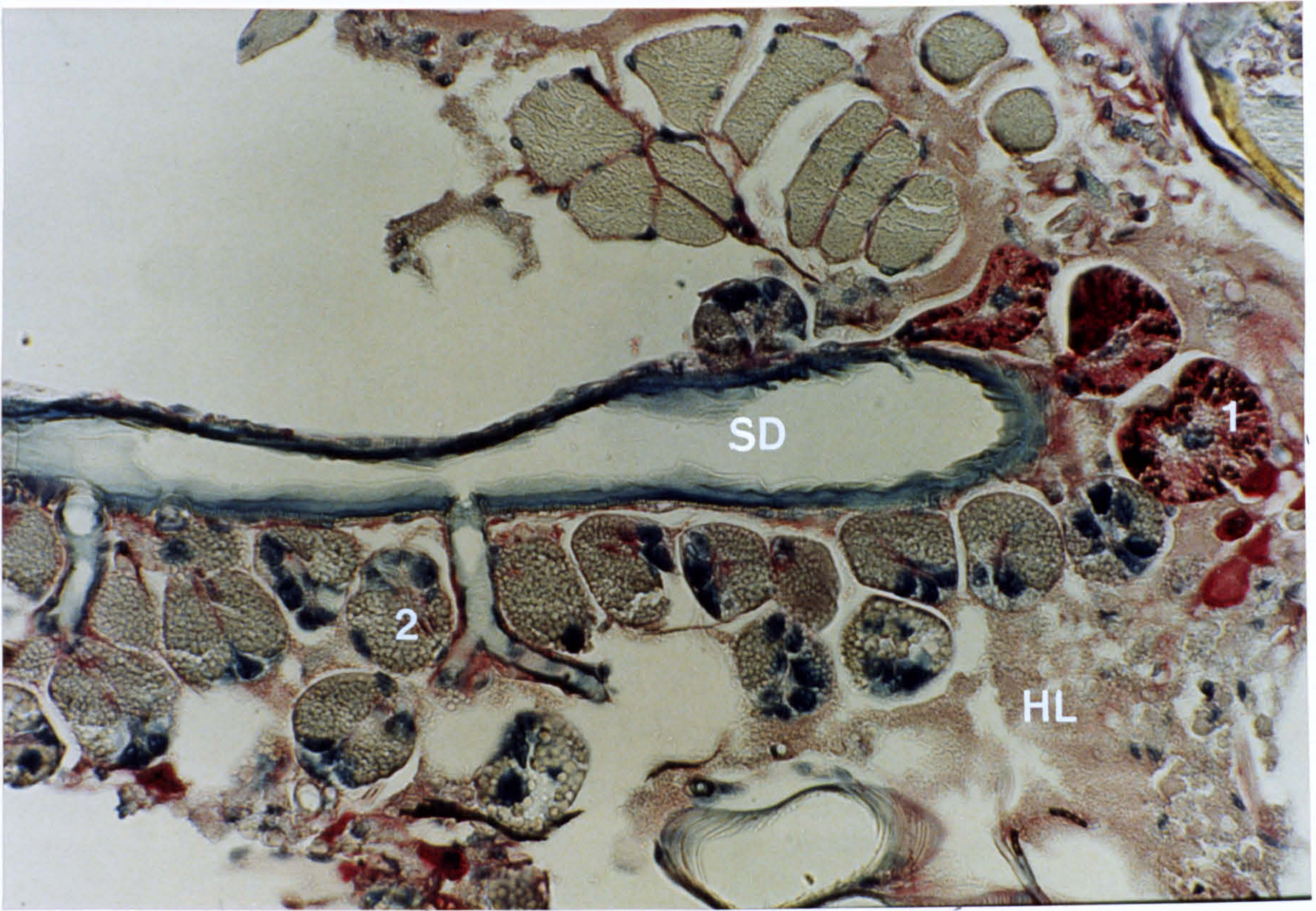


g

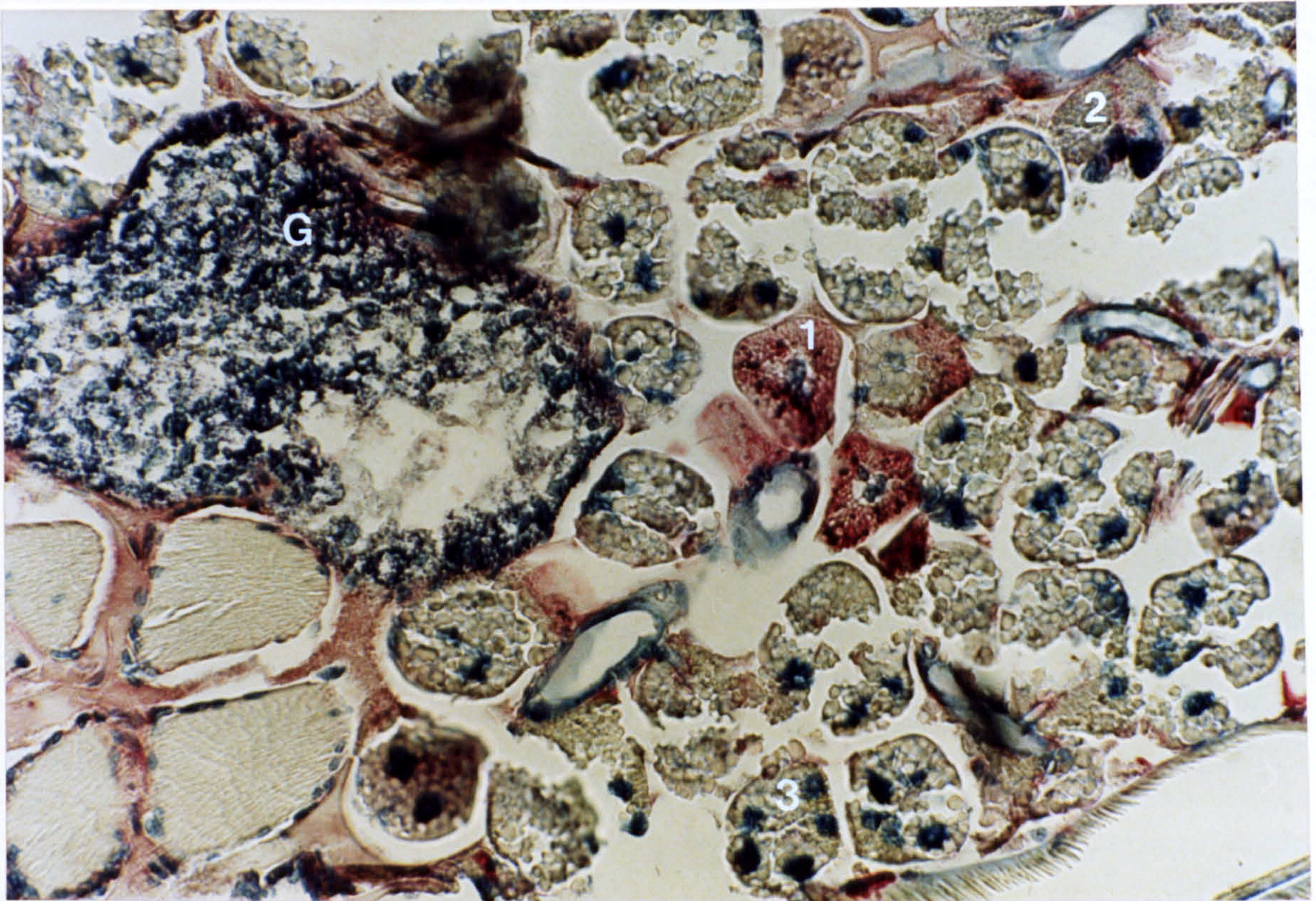


h

i,j: Tissues Stained using Guinea Pig Antiserum against *Rh. appendiculatus* Unfed Nymphal Extract 45% Ammonium Sulphate Supernate Fraction (Magnification x100).

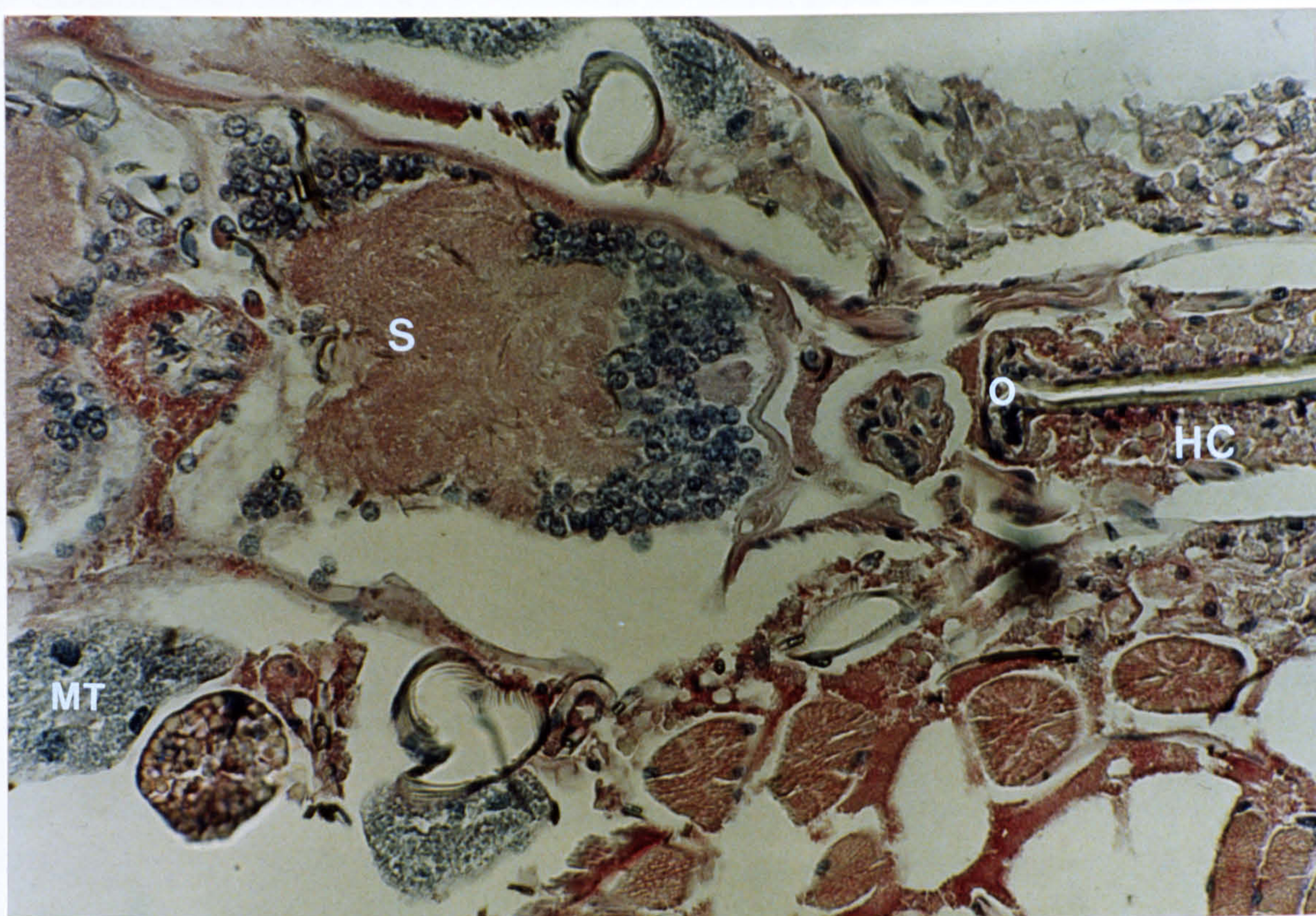


i

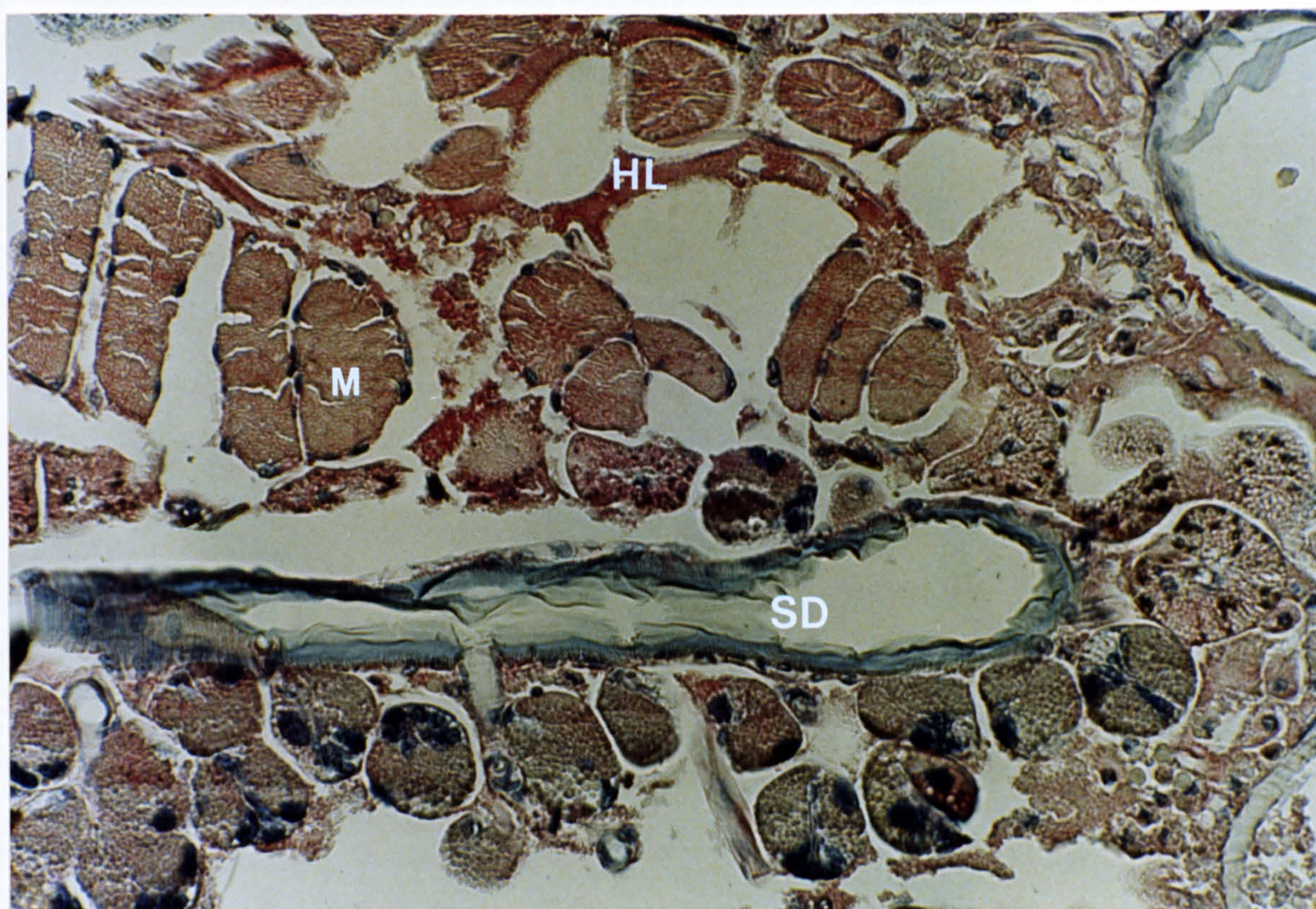


j

k,l: Tissues Stained using Guinea Pig Antiserum against *Rh. appendiculatus* Unfed Nymphal Extract 45% Ammonium Sulphate Supernate Fraction, reduced dose (135µg) (Magnification x100).



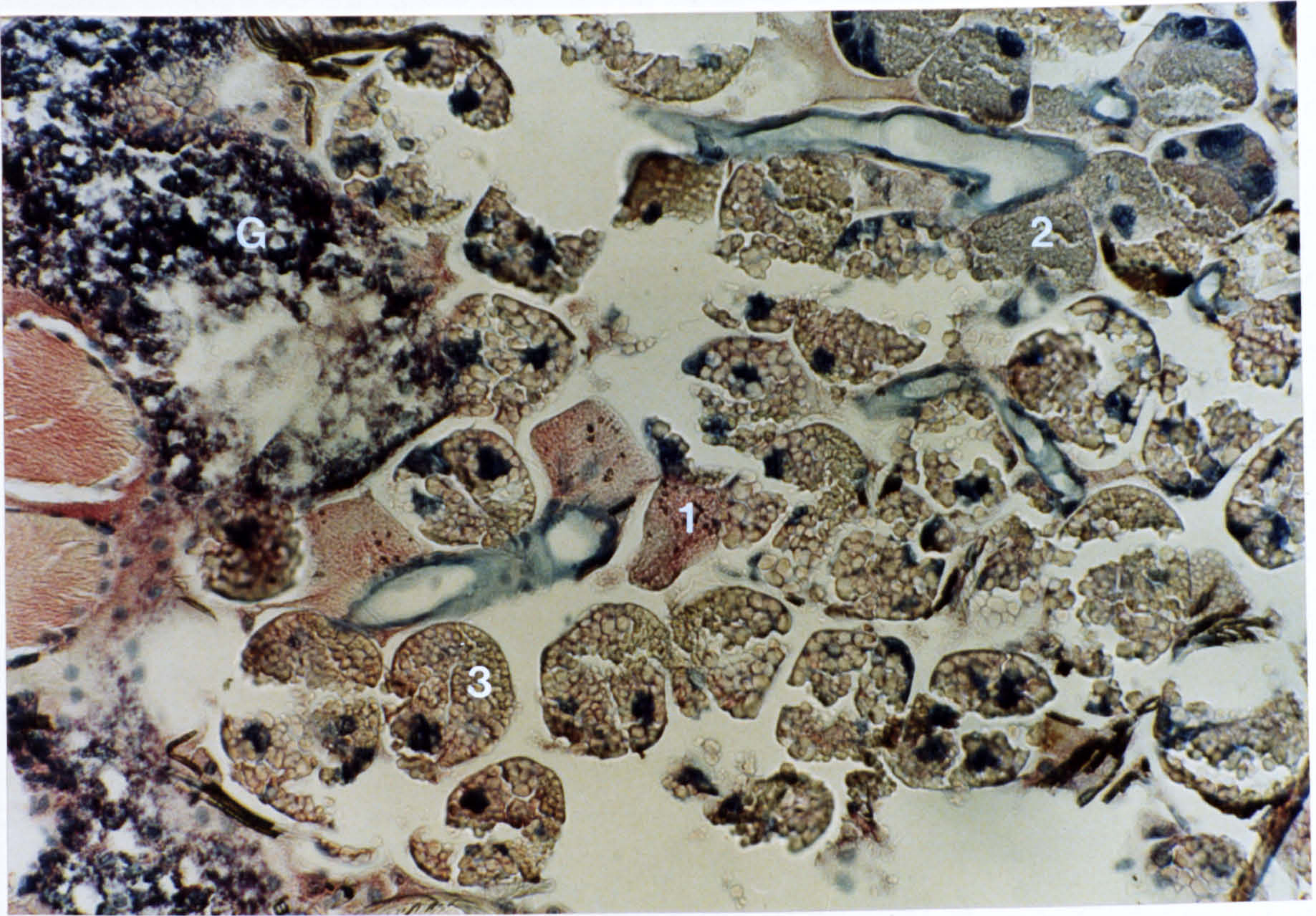
k



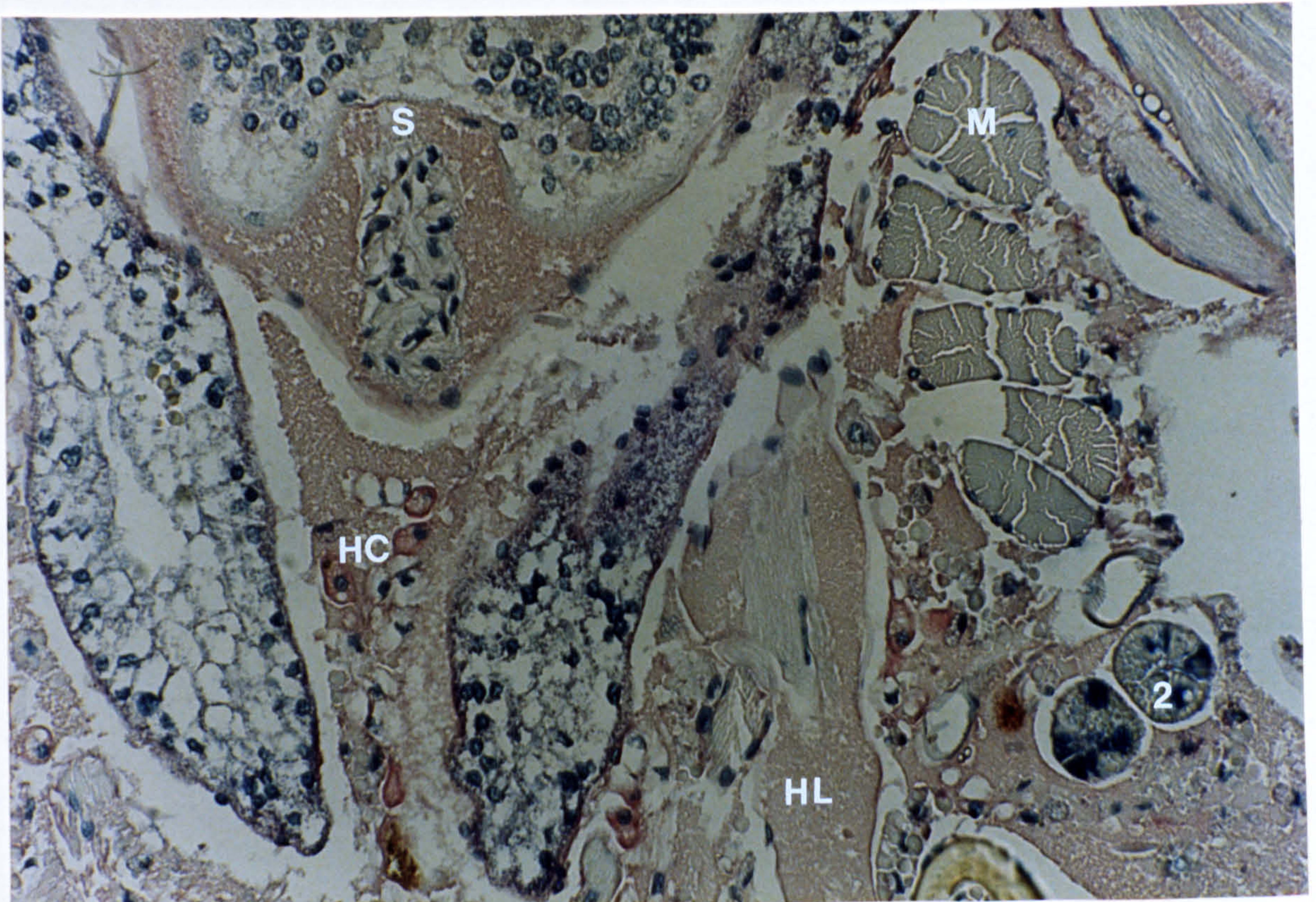
l

m: Tissues Stained using Guinea Pig Antiserum against *Rh. appendiculatus* Unfed Nymphal Extract 45% Ammonium Sulphate Supernate Fraction, reduced dose (135µg) (Magnification x100).

n: Tissues Stained using Guinea Pig Antiserum against *Rh. appendiculatus* Unfed Nymphal Extract SEHPLC Fraction 2 (Magnification x100).



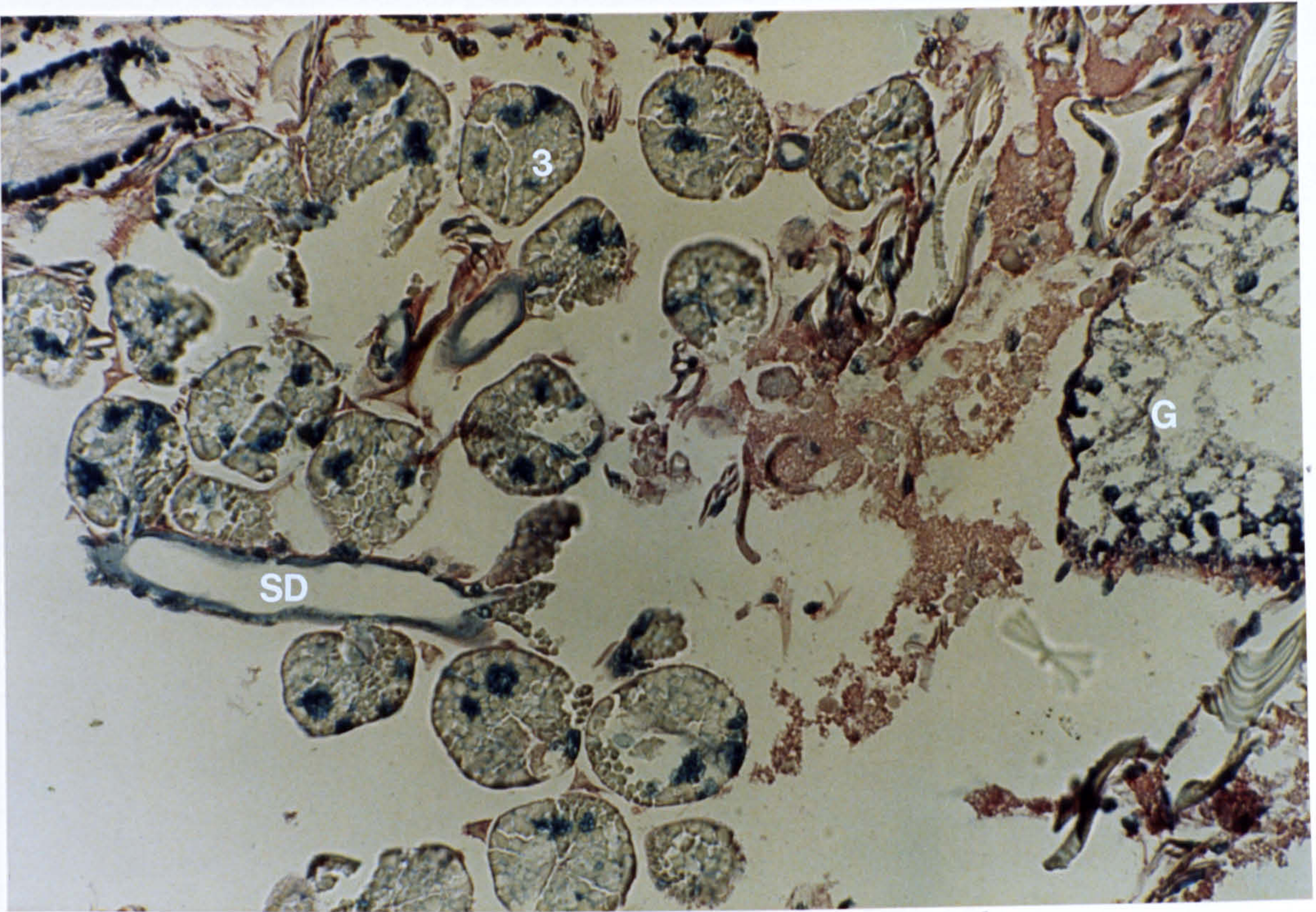
m



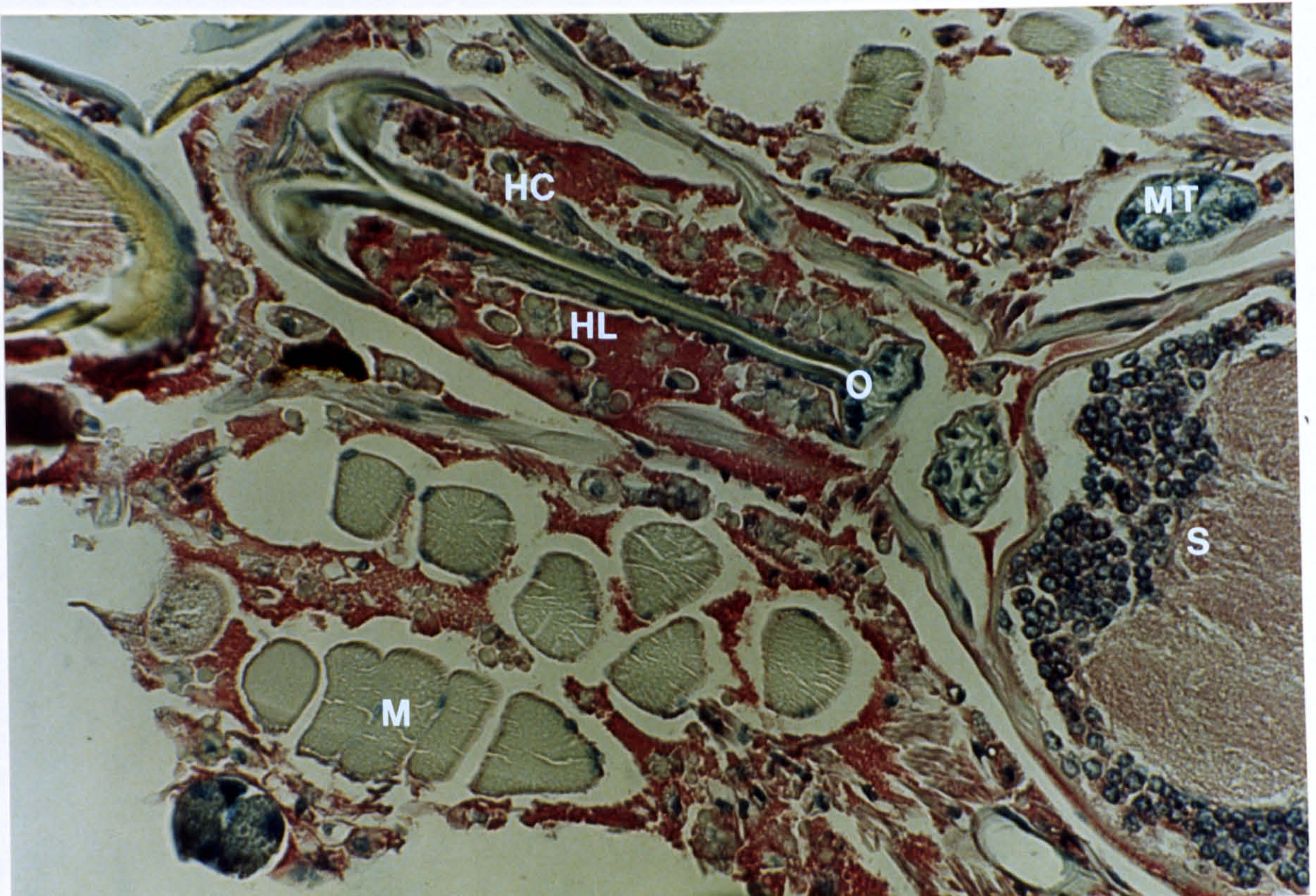
n

o: Tissues Stained using Guinea Pig Antiserum against *Rh. appendiculatus* Unfed Nymphal Extract SEHPLC Fraction 2 (Magnification x100).

p: Tissues Stained using Guinea Pig Antiserum against Nitrocellulose Particles Bearing Antigens from *Rh. appendiculatus* Unfed Nymphal Extract (Magnification x100).

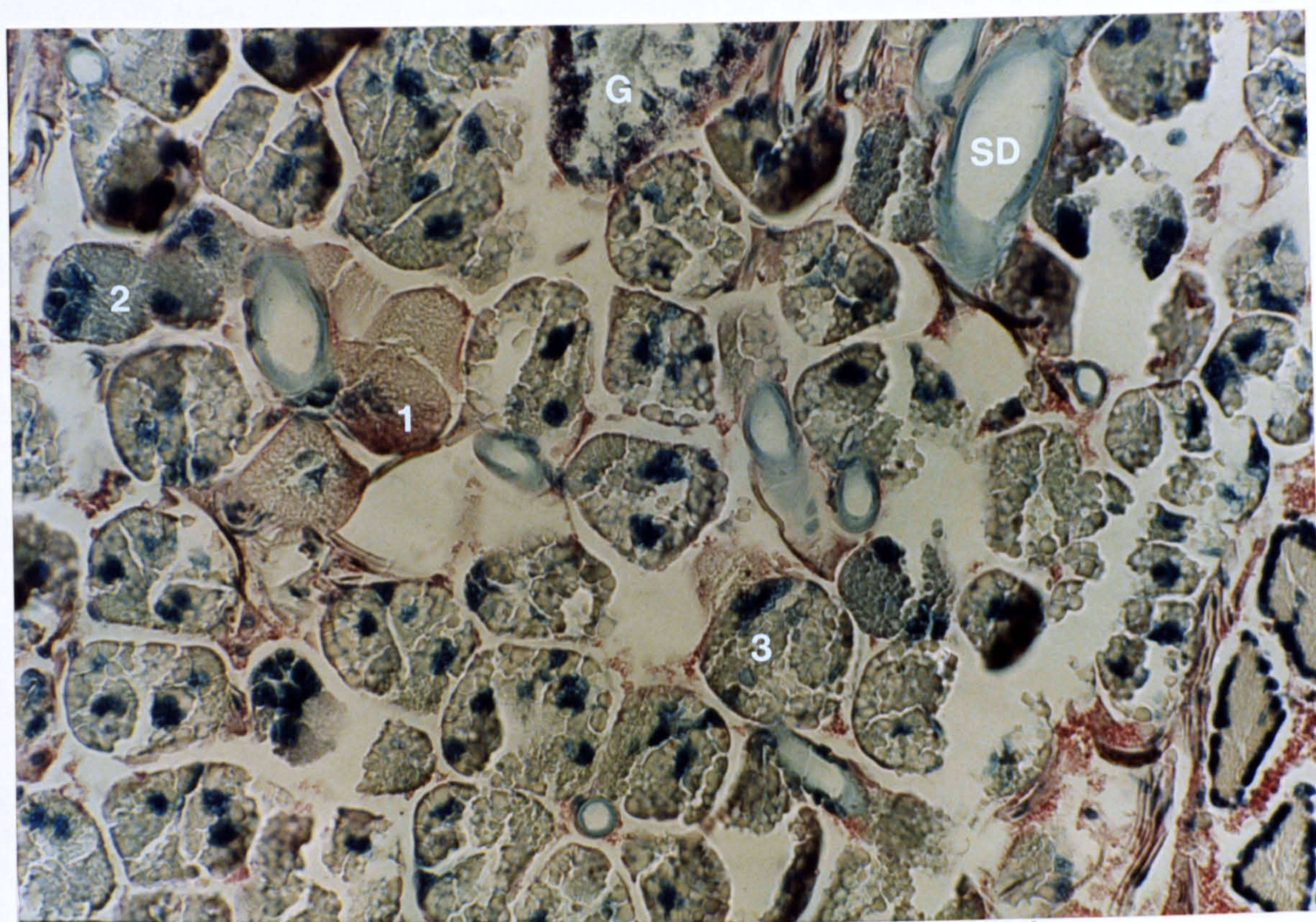


0



p

q: Tissues Stained using Guinea Pig Antiserum against Nitrocellulose Particles Bearing Antigens from *Rh. appendiculatus* Unfed Nymphal Extract (Magnification x100).



q

3.6 Immunisation of Guinea Pigs and Challenge by *Rh. appendiculatus* Adult Tick Feed.

Of the various parameters for assessing feeding and reproductive performance of female *Rh. appendiculatus* on immunised and control guinea pigs, engorged female weight and weight of egg masses were the most consistent. In the present studies, I have therefore used significant reductions of these two parameters in ticks feeding on immunised animals for assessing protective immunity. There were no significant differences in other parameters, except where they are mentioned in the text. Similar numbers (6 - 10) of fed female ticks were recovered from immunised and control animals. Average feeding times of females from both groups were similar, 7.5 ± 0.8 to 9.9 ± 1.8 days for ticks from immunised animals and 8.5 ± 1.2 to 10.7 ± 1.9 days for ticks from the control groups. There was no consistent pattern in the conversion efficiency index (CEI) which is a measure of the ability of the females to convert blood meal into eggs and expressed as the percentage of the egg mass weight in relation to blood meal weight. The CEI ranged from $26.1 \pm 5\%$ to $50.9 \pm 7.3\%$ for ticks from immunised animals and $34.0 \pm 1.4\%$ to $37.7 \pm 1.3\%$ for ticks from control animals; in some cases ticks from immunised animals had a higher CEI than those from control animals. There were also extreme variations in egg hatch rates. Rates of less than 5% and upto 100% were seen in eggs from both groups of guinea pigs.

3.6.1. Immunisation with Whole *Rh. appendiculatus* Unfed Nymphal Extract or Gut Extract from Partially Engorged Female Ticks.

Groups of three guinea pigs were immunised with *Rh. appendiculatus* unfed nymphal extract (UNE) or gut extract (for antigen profile see 3.5.1.4) mixed with an equal volume of Freund's incomplete adjuvant (FIA) at the doses indicated in table 3.16. Three control guinea pigs received FIA only.

Upon challenge feed with *Rh. appendiculatus* adults, the gut extract reduced engorged female weight ($290.62\text{mg} \pm 20.10\text{mg}$) by just over 29% ($P < 0.01$) when compared to engorged females from the FIA control group ($410.01\text{mg} \pm 21.80\text{mg}$). The resultant egg mass ($106.57\text{mg} \pm 9.22\text{mg}$) was more than 26.5% ($P < 0.01$) lighter than that from control ticks ($145.24\text{mg} \pm 8.50\text{mg}$). Engorged ticks from the UNE group ($154.92\text{mg} \pm 28.01\text{mg}$) were actually 62% ($P < 0.001$) lighter than ticks from the control group, and there was a corresponding reduction in egg mass weight ($57.72\text{mg} \pm 11.43\text{mg}$) of more than 60% ($P < 0.001$). When compared to the gut extract group, the UNE reduced engorged tick weight by 52.3% more than the gut extract ($P < 0.05$).

3.6.2. Immunisation with Whole *Rh. appendiculatus* Unfed Larval Extract and Its Fractions (Various Treatments).

Two guinea pigs were immunised with *Rh. appendiculatus* unfed larval extract, and further groups of two guinea pigs were immunised with the same extract after charcoal treatment,

TABLE 3.16 RESULTS OF *RH. APPENDICULATUS* ADULT CHALLENGE FEED ON GUINEA PIGS IMMUNISED WITH WHOLE UNFED NYMPHAL EXTRACT AND GUT EXTRACT FROM PARTIALLY ENGORGED FEMALE TICKS.

MATERIAL INOCULATED	FEMALE ENGORGED WEIGHT (mg) (MEAN \pm SE)	REDUCTION IN ENGORGED WEIGHT (%)	WEIGHT OF EGG MASS (mg)	REDUCTION IN EGG MASS WEIGHT (%)
FIA (control)	410.01 ^A \pm 21.80	-	145.24 \pm 8.50	-
UNE	154.92 ^B \pm 28.01	62.22 (P<0.001)	57.72 \pm 11.43	60.26 (P<0.001)
Gut Extract	290.62 ^C \pm 20.10	29.12 (P<0.01)	106.57 \pm 9.22	26.62 (P<0.01)

SE Standard Error of Mean FIA Freund's Incomplete Adjuvant
UNE Unfed Nymphal Extract * Figures with dissimilar superscripts are significantly different (P<0.05)

TABLE 3.17 RESULTS OF *RH. APPENDICULATUS* ADULT CHALLENGE FEED ON GUINEA PIGS IMMUNISED WITH WHOLE UNFED LARVAL EXTRACT AND ITS FRACTIONS (VARIOUS TREATMENTS).

MATERIAL INOCULATED	FEMALE ENGORGED WEIGHT (mg) (MEAN \pm SE)	REDUCTION IN ENGORGED WEIGHT (%)	WEIGHT OF EGG MASS (mg)	REDUCTION IN EGG MASS (%)
FIA	464.35 ^{A*} ± 20.63	-	163.45 ± 10.85	-
ULE	320.77 ^{BC} ± 35.34	30.92 (P<0.001)	73.91 ± 17.12	54.78 (P<0.001)
Charcoal treated ULE	271.95 ^C ± 20.63	41.43 (P<0.001)	84.16 ± 12.14	48.51 (P<0.001)
Dialysed ULE	366.38 ^B ± 20.94	21.10 (P<0.01)	110.16 ± 14.19	32.61 (P<0.01)
ULE PEG 8000 supernate	375.14 ^B ± 16.87	19.21 (P<0.01)	106.44 ± 8.57	34.88 (P<0.001)
ULE ammonium sulphate precipitate	337.73 ^{BC} ± 41.61	27.27 (P<0.01)	106.77 ± 16.22	34.68 (P<0.01)
ULE ammonium sulphate supernate	284.97 ^C ± 34.74	38.63 (P<0.01)	94.91 ± 11.75	41.93 (P<0.01)

SE Standard Error of Mean FIA Freund's Incomplete Adjuvant
ULE Unfed Larval Extract * Figures with dissimilar superscripts
are significantly different (P<0.05)

dialysis, precipitation with PEG 8000 (supernate), or precipitation with saturated ammonium sulphate (precipitate and supernate) (for antigen profiles refer 3.5.2.1). In all cases the dose rate was 3.0mg.kg^{-1} body weight, and extracts were mixed with equal volumes of FIA. Two control guinea pigs received FIA only (table 3.17).

When compared to *Rh. appendiculatus* females fed on control animals, females fed on ULE immunised guinea pigs were found to be nearly 31% ($P < 0.001$) lighter ($320.77\text{mg} \pm 35.34\text{mg}$), and the resultant egg mass ($73.91\text{mg} \pm 17.12\text{mg}$) reduced in weight by almost 55% ($P < 0.001$) compared to that of control ticks ($464.35\text{mg} \pm 20.63\text{mg}$ and $163.45\text{mg} \pm 10.85\text{mg}$, female engorged weight and egg mass weight respectively). The reduction in engorged tick weight ($271.95\text{mg} \pm 20.63\text{mg}$) in the charcoal washed UNE group was just under 41.5% ($P < 0.001$), and the weight of the resulting egg mass ($84.16\text{mg} \pm 12.14\text{mg}$) was reduced by slightly less than 48.5% ($P < 0.001$); for ticks from the dialysed UNE group the figures were somewhat less, being just over 21% ($P < 0.01$) and 32.6% ($P < 0.01$) ($366.38\text{mg} \pm 20.94\text{mg}$ and $110.16\text{mg} \pm 14.19\text{mg}$) respectively. Material in the PEG 8000 supernate induced an engorged tick weight ($375.14\text{mg} \pm 16.87\text{mg}$) reduction of just over 19% ($P < 0.001$) and the egg mass weight ($106.44\text{mg} \pm 8.57\text{mg}$) was reduced by nearly 35% ($P < 0.01$). For the ammonium sulphate precipitate, the figures were just under 27.5% ($P < 0.01$) for tick weight ($337.73\text{mg} \pm 41.61\text{mg}$) reduction, and just over 34.5% ($P < 0.01$) for reduction in

egg mass weight ($106.77\text{mg} \pm 16.22\text{mg}$); these compared to more than 38.5% ($P < 0.01$) ($284.97\text{mg} \pm 34.74\text{mg}$) and just under 42% ($P < 0.01$) ($94.91\text{mg} \pm 11.75\text{mg}$) respectively for the ammonium sulphate supernate. When compared to the ULE group, none of the fractions were found to be significantly better ($P > 0.05$) at reducing engorged tick weight. However, the ammonium sulphate supernate and the charcoal treated ULE were both found to be significantly better ($P < 0.05$) at reducing engorged tick weight than all the other fractions immunised.

3.6.3 Immunisation with Whole *Rh. appendiculatus* Unfed Nymphal Extract and Its SEHPLC Fractions.

UNE was fractionated by SEHPLC and groups of two guinea pigs vaccinated as in table 3.18.

Compared to ticks ($397.92\text{mg} \pm 24.01\text{mg}$) fed on control animals (FIA only), engorged *Rh. appendiculatus* females ($178.91\text{mg} \pm 41.88\text{mg}$) recovered from guinea pigs vaccinated with whole UNE showed a reduction in weight in excess of 55% ($P < 0.001$). SEHPLC fraction 1 induced an engorged tick weight ($266.04\text{mg} \pm 18.91\text{mg}$) reduction of just over 33% ($P < 0.001$); whilst fraction 2 brought about a 68% ($P < 0.001$) reduction in tick weight ($127.33\text{mg} \pm 27.46\text{mg}$). Ticks from the fraction 3 group ($305.87\text{mg} \pm 37.55\text{mg}$) were found to be more than 23% ($P < 0.05$) lighter than engorged control ticks. Egg mass weights were not determined for this

TABLE 3.18 RESULTS OF *RH. APPENDICULATUS* ADULT CHALLENGE FEED ON GUINEA PIGS IMMUNISED WITH WHOLE UNFED NYMPHAL EXTRACT AND ITS SEHPLC FRACTIONS (1st. Experiment).

MATERIAL INOCULATED	FEMALE ENGORGED WEIGHT (mg) (MEAN \pm SE)	REDUCTION IN ENGORGED WEIGHT (Z)	WEIGHT OF EGG MASS (mg)	REDUCTION IN EGG MASS WEIGHT (Z)
FIA	397.92 ^{A*} ± 24.01	-	-	-
UNE	178.91 ^C ± 41.88	55.04 (P<0.001)	-	-
UNE SEHPLC fraction 1	266.04 ^B ± 18.91	33.14 (P<0.001)	-	-
UNE SEHPLC fraction 2	127.33 ^C ± 27.46	68.00 (P<0.001)	-	-
UNE SEHPLC fraction 3	305.87 ^{AB} ± 37.55	23.13 (P<0.05)	-	-

SE Standard Error of Mean FIA Freund's Incomplete Adjuvant

UNE Unfed Nymphal Extract SEHPLC Size Exclusion HPLC

* Figures with dissimilar superscripts are significantly different (P<0.05)

TABLE 3.19 RESULTS OF *RH. APPENDICULATUS* CHALLENGE FEED ON GUINEA PIGS IMMUNISED WITH WHOLE UNFED NYMPHAL EXTRACT AND ITS SEHPLC FRACTIONS (2nd. Experiment).

MATERIAL INOCULATED	FEMALE ENGORGED WEIGHT (mg) (MEAN ± SE)	REDUCTION IN ENGORGED WEIGHT (%) (P<0.05)	WEIGHT OF EGG MASS (mg) (±12.58)	REDUCTION IN EGG MASS WEIGHT (%) (P<0.8)
FIA	335.81 ^{A*} ±28.13	-	127.42 ±12.58	-
UNE	257.58 ^B ±24.65	23.30 (P<0.05)	121.45 ±8.77	4.69 (P<0.8)
UNE SEHPLC fraction 1	255.43 ^B ±21.93	23.94 (P<0.05)	96.90 ±7.87	26.42 (P<0.02)
UNE SEHPLC fraction 2	118.51 ^C ±20.59	64.71 (P<0.001)	61.29 ±9.78	51.90 (P<0.001)
UNE SEHPLC fraction 3	287.36 ^B ±22.55	14.43 (P<0.2)	127.00 ±9.97	0.33 (P<0.8)

SE Standard Error of Mean FIA Freunds Incomplete Adjuvant
UNE Unfed Nymphal Extract SEHPLC Size Exclusion HPLC
* Figures with dissimilar superscripts are significantly different (P<0.05)

TABLE 3.20 RESULTS OF *RH. APPENDICULATUS* ADULT CHALLENGE FEED ON GUINEA PIGS IMMUNISED WITH WHOLE UNFED NYMPHAL EXTRACT AND ITS SEHPLC FRACTIONS (3rd. Experiment).

MATERIAL INOCULATED	FEMALE ENGORGED WEIGHT (mg) (MEAN \pm SE)	REDUCTION IN ENGORGED WEIGHT (Z)	WEIGHT OF EGG MASS (mg)	REDUCTION IN EGG MASS WEIGHT (Z)
FIA	448.64 ^{A*} ± 17.84	-	-	-
UNE	310.37 ^{BC} ± 26.86	30.82 (P<0.001)	-	-
UNE SEHPLC fraction 2	389.98 ^C ± 16.63	13.08 (P<0.05)	-	-
UNE SEHPLC fraction 3	286.64 ^B ± 23.07	36.23 (P<0.001)	-	-

SE Standard Error of Mean FIA Freund's Incomplete Adjuvant

UNE Unfed Nymphal Extract SEHPLC Size Exclusion HPLC

* Figures with dissimilar superscripts are significantly different (P<0.05)

experiment. The UNE and its fraction 2 were both found to be significantly better ($P < 0.05$) than fractions 1 and 3 at reducing engorged tick weight. There was no significant difference between the values obtained for the UNE and fraction 2 ($P > 0.05$).

In a repeat experiment, using the same batch of UNE and a fresh fractionation, and three guinea pigs *per* group, the following results were obtained (table 3.19) (antigen profiles 3.5.2.2).

The UNE induced a reduction of just under 23.5% ($P < 0.05$) in engorged tick weight, compared to the control inoculation ($257.58\text{mg} \pm 24.65\text{mg}$ compared to $335.81\text{mg} \pm 28.13\text{mg}$), and a reduction of only just over 4.5% (not significant $P = 0.8$) in egg mass ($121.45\text{mg} \pm 8.77\text{mg}$ compared to $127.42\text{mg} \pm 12.58\text{mg}$). These compared with figures of nearly 24% ($P < 0.05$) and just under 26.5% ($P < 0.02$), tick weight ($255.43\text{mg} \pm 21.93\text{mg}$) and egg mass weight ($96.9\text{mg} \pm 7.87\text{mg}$) respectively, for the fraction 1 group. Fraction 2 caused a reduction of more than 64.5% ($P < 0.001$) in tick weight ($118.51\text{mg} \pm 20.59\text{mg}$), and of these fed ticks less than 58% (15 out of 26) laid eggs (highly significant, $P < 0.001$), with a reduction of 52% ($P < 0.001$) in egg mass weight ($61.29\text{mg} \pm 9.78\text{mg}$) *per* tick compared to controls. Of the eggs laid, only 38% ($P < 0.01$) hatched. The tick weight ($287.36\text{mg} \pm 22.55\text{mg}$) reduction in the fraction 3 group was just under 14.5% ($P = 0.2$) with an accompanying egg mass weight ($127.00\text{mg} \pm 9.97\text{mg}$) reduction of less than 0.5% (not significant, $P = 0.8$). Fraction 2

was found to be significantly better ($P < 0.05$) than fractions 1 and 3 and the UNE at reducing engorged tick weight. The reduction induced by fraction 2 when compared to the UNE was 54%.

Using a different UNE, a further SEHPLC fractionation was carried out and groups of three guinea pigs immunised against fractions 2 and 3 with doses equivalent to the percentage protein content based on UNE = 100% (table 3.20) (antigen profiles 3.5.2.2).

Engorged ticks ($310.37\text{mg} \pm 26.86\text{mg}$) from the UNE group were nearly 31% lighter than ticks from the control group ($448.64\text{mg} \pm 17.84\text{mg}$), whilst ticks ($389.98\text{mg} \pm 16.63\text{mg}$) from the fraction 2 group were only just over 13% lighter. A weight reduction of >36% was observed in ticks ($286.64\text{mg} \pm 23.07\text{mg}$) fed on animals vaccinated with fraction 3. Weights of egg masses were not determined for this experiment. When compared to the UNE group, neither of the fractions was found to be significantly better ($P > 0.05$) than the UNE in reducing engorged tick weight. However, fraction 3 was significantly better than fraction 2 ($P < 0.05$) at reducing engorged tick weight.

3.6.4. Immunisation with Whole *Rh. appendiculatus* Unfed Nymphal Extract and Its Ammonium Sulphate Partitioned Fractions.

Groups of three guinea pigs were inoculated as in table 3.21. The UNE used was from the same batch as in tables 3.18 and 3.19.

TABLE 3.21 RESULTS OF *RH. APPENDICULATUS* ADULT CHALLENGE FEED ON GUINEA PIGS IMMUNISED WITH WHOLE UNFED NYMPHAL EXTRACT AND ITS AMMONIUM SULPHATE PARTITIONED FRACTIONS (1st. Experiment).

MATERIAL INOCULATED	FEMALE ENGORGED WEIGHT (mg) (MEAN \pm SE)	REDUCTION IN ENGORGED WEIGHT (%)	WEIGHT OF EGG MASS (mg)	REDUCTION IN EGG MASS WEIGHT (%)
FIA	446.57 ^{A*} ± 14.73	-	-	-
UNE	233.69 ^C ± 23.90	47.67 (P<0.001)	-	-
UNE 20% ammonium sulphate precipitate	322.40 ^B ± 17.86	27.81 (P<0.001)	-	-
UNE 35% ammonium sulphate precipitate	296.06 ^B ± 25.04	33.70 (P<0.001)	-	-
UNE 45% ammonium sulphate precipitate	328.06 ^B ± 20.67	26.54 (P<0.001)	-	-
UNE 45% ammonium sulphate supernate	199.37 ^C ± 26.69	55.36 (P<0.001)	-	-

SE Standard Error of Mean FIA Freund's Incomplete Adjuvant
 UNE Unfed Nymphal Extract * Figures with dissimilar superscripts
 are significantly different (P<0.05)

TABLE 3.22 RESULTS OF *RH. APPENDICULATUS* ADULT CHALLENGE FEED ON GUINEA PIGS IMMUNISED WITH WHOLE UNFED NYMPHAL EXTRACT AND ITS AMMONIUM SULPHATE PARTITIONED FRACTIONS (2nd. Experiment).

MATERIAL INOCULATED	FEMALE ENGORGED WEIGHT (mg) (MEAN \pm SE)	REDUCTION IN ENGORGED WEIGHT (%)	WEIGHT OF EGG MASS (mg)	REDUCTION IN EGG MASS WEIGHT (%)
FIA	405.24 ^A \pm 14.18	-	156.81 \pm 8.12	-
UNE	250.44 ^B \pm 19.86	38.20 (P<0.001)	105.60 \pm 8.53	32.66 (P<0.001)
UNE 20% ammonium sulphate precipitate	251.90 ^B \pm 27.62	37.84 (P<0.001)	-	-
UNE 35% ammonium sulphate precipitate	253.94 ^B \pm 23.67	37.34 (P<0.001)	-	-
UNE 45% ammonium sulphate precipitate	273.67 ^B \pm 21.41	32.47 (P<0.001)	105.81 \pm 9.68	32.52 (P<0.001)
UNE 45% ammonium sulphate supernate	155.07 ^C \pm 28.34	61.73 (P<0.001)	74.04 \pm 12.37	52.78 (P<0.001)

SE Standard Error of Mean FIA Freund's Incomplete Adjuvant
 UNE Unfed Nymphal Extract * Figures with dissimilar superscripts
 are significantly different (P<0.05)

TABLE 3.23 RESULTS OF *RH. APPENDICULATUS* ADULT CHALLENGE FEED ON GUINEA PIGS IMMUNISED WITH WHOLE UNFED NYMPHAL EXTRACT AND ITS AMMONIUM SULPHATE PARTITIONED FRACTIONS (3rd. Experiment).

MATERIAL INOCULATED	FEMALE ENGORGED WEIGHT (mg) (MEAN ± SE)	REDUCTION IN ENGORGED WEIGHT (%) (P<0.001)	WEIGHT OF EGG MASS (mg) (P<0.001)	REDUCTION IN EGG MASS WEIGHT (%) (P<0.02)
FIA	314.92 ^A ±23.18	-	114.41 ±9.49	-
UNE (dose = 3 x 450 = 1350 µg)	188.02 ^{BC} ±17.82	40.30 (P<0.001)	82.63 ±9.02	27.8 (P<0.02)
UNE (dose = 3 x 45 = 135 µg)	215.45 ^B ±20.49	31.59 (P<0.01)	92.49 ±7.66	19.2 (P<0.10)
UNE 45% ammonium sulphate precipitate (3 x 450 = 1350 µg)	217.71 ^B ±30.53	30.87 (P<0.02)	77.14 ±8.54	32.6 (P<0.01)
UNE 45% ammonium sulphate supernate (3 x 450 = 1350 µg)	170.70 ^{BC} ±21.60	45.80 (P<0.001)	71.52 ±7.43	37.5 (P<0.001)
UNE 45% ammonium sulphate supernate (3 x 45 = 135 µg)	140.99 ^C ±23.26	55.23 (P<0.001)	65.69 ±9.78	42.6 (P<0.001)

SE Standard Error of Mean FIA Freund's Incomplete Adjuvant
 UNE Unfed Nymphal Extract * Figures with dissimilar superscripts are significantly different (P<0.05)

When compared to the weight of engorged ticks ($446.57\text{mg} \pm 14.73\text{mg}$) from the control group, the UNE reduced engorged tick weight ($233.69\text{mg} \pm 23.90\text{mg}$) by more than 47.5% ($P < 0.001$), whilst the 20% (w/v) ammonium sulphate precipitate induced a weight ($322.40\text{mg} \pm 17.86\text{mg}$) reduction of just under 28% ($P < 0.001$). A tick weight ($296.06\text{mg} \pm 25.04\text{mg}$) reduction in excess of 33.5% ($P < 0.001$) was observed in the 35% ammonium sulphate precipitate group, and with the 45% precipitate the tick weight ($328.06\text{mg} \pm 20.67\text{mg}$) reduction was just over 26.5% ($P < 0.001$). A weight reduction of nearly 55.5% ($P < 0.001$) was observed in ticks ($199.37\text{mg} \pm 26.69\text{mg}$) from guinea pigs inoculated with the 45% supernate. Weights of egg masses were not determined for this experiment. The UNE and its 45% supernate fraction were found to be significantly better ($P < 0.05$) than the three precipitate fractions at reducing engorged tick weight. There was no significant difference between the values obtained for the UNE and the 45% supernate ($P > 0.05$).

In a repeat experiment involving a different batch of extract (table 3.22), the UNE reduced engorged tick weight ($250.44\text{mg} \pm 19.86\text{mg}$) by 38% ($P < 0.001$), compared to ticks ($405.24\text{mg} \pm 14.18\text{mg}$) in the control group, and the egg mass weight ($105.60\text{mg} \pm 8.53\text{mg}$ compared with $156.81\text{mg} \pm 8.12\text{mg}$ for eggs from control ticks) was reduced by over 32.5% ($P < 0.001$). The 20% precipitate reduced the tick weight ($251.90\text{mg} \pm 27.62\text{mg}$) by nearly 38% ($P < 0.001$) and the 35% precipitate by just under 37.5% ($P < 0.001$) ($253.94\text{mg} \pm 23.67\text{mg}$); weights of egg masses were not determined for these two groups. Inoculation with the 45% precipitate induced a tick

weight ($273.67\text{mg} \pm 21.41\text{mg}$) reduction of slightly less than 32.5% ($P < 0.001$), and an egg mass weight ($105.81\text{mg} \pm 9.68\text{mg}$) reduction of just over 32.5%; these compared with reductions for the ammonium sulphate 45% supernate of more than 61.5% ($P < 0.001$) and nearly 53% ($P < 0.001$) tick weight ($155.07\text{mg} \pm 28.34\text{mg}$) and egg mass weight ($74.04\text{mg} \pm 12.37\text{mg}$) respectively (antigen profiles 3.5.2.3). The 45% supernate fraction was found to be significantly better ($P < 0.05$) than the other fractions and the UNE at reducing engorged tick weight. The reduction induced by the 45% supernate when compared to the UNE was 38.1%.

The effects of reducing the dose, *per* guinea pig, of inoculum were investigated (table 3.23, three guinea pigs *per* group). UNE, inoculated with a total of $1350\mu\text{g}$ protein.guinea pig⁻¹, induced a reduction in engorged tick weight ($188.02\text{mg} \pm 17.82\text{mg}$) of over 40% ($P < 0.001$) compared to the control group (tick weight 314.92 ± 23.18), whilst the egg mass weight was reduced by nearly 28% ($P = 0.02$) ($82.63\text{mg} \pm 9.02\text{mg}$ compared to $114.41 \pm 9.49\text{mg}$ for the control group). These reductions compared with just over 31.5% ($P < 0.01$) and more than 19% ($P < 0.01$), tick weight ($215.45\text{mg} \pm 20.49\text{mg}$) and egg mass weight ($92.49\text{mg} \pm 7.66\text{mg}$) respectively, achieved with UNE at a total dose of $135\mu\text{g}$.guinea pig⁻¹). The 45% precipitate (total of $1350\mu\text{g}$.guinea pig⁻¹) gave a tick weight ($217.71\text{mg} \pm 30.53\text{mg}$) reduction of nearly 31% ($P < 0.02$) and a reduction in egg mass weight ($77.14\text{mg} \pm 8.54\text{mg}$) of 32.5% ($P < 0.01$). These compared with reductions of nearly 46% ($P < 0.001$) in tick weight ($170.70\text{mg} \pm 21.60\text{mg}$) and nearly 37.5% ($P < 0.001$) in

egg mass weight ($71.52\text{mg} \pm 7.43\text{mg}$) when guinea pigs were inoculated with 45% supernate at a similar dose. Interestingly when the dose of 45% supernate was reduced to $135\mu\text{g.guinea pig}^{-1}$, reductions of just over 55% ($P<0.001$) in tick weight ($140.99\text{mg} \pm 23.26\text{mg}$) and of more than 42.5% ($P<0.001$) in egg mass weight ($65.69\text{mg} \pm 9.78\text{mg}$) were achieved (antigen profile 3.5.2.3). A similar dose of crude UNE gave a reduction in engorged weight of about 32% (see above). The 45% supernate ($135\mu\text{g}$) was found to be significantly better ($P<0.05$) at reducing engorged tick weight than the 45% precipitate ($1350\mu\text{g}$) and the UNE ($135\mu\text{g}$). However, there was no significant difference ($P>0.05$) between the 45% supernate ($135\mu\text{g}$), 45% supernate ($1350\mu\text{g}$) and the UNE ($1350\mu\text{g}$). The reduction induced by the 45% supernate ($135\mu\text{g}$) when compared to the UNE ($135\mu\text{g}$) was 34.6%.

3.6.5 Immunisation with Whole *Rh. appendiculatus* Unfed Nymphal Extract and an Electrophoretically Isolated Antigen Fraction.

Nitrocellulose particles with antigens covalently attached (see 2.4.2) were inoculated as in table 3.24. The whole UNE gave a reduction in engorged tick weight ($303.00\text{mg} \pm 27.17\text{mg}$) of 32% ($P<0.001$) compared to ticks ($445.68\text{mg} \pm 18.21\text{mg}$) from the control group, and an egg mass weight ($115.62\text{mg} \pm 11.75\text{mg}$) reduction of just over 23.5% ($P<0.05$) compared to controls ($151.22\text{mg} \pm 8.75\text{mg}$). Tick weight ($269.62\text{mg} \pm 27.10\text{mg}$) was reduced by 39.5% ($P<0.001$) in the nitrocellulose-antigen group; of these fed ticks 26% (7 out of 27) did not lay eggs (significant, $P<0.02$), and

those that did produce eggs had egg masses ($123.76\text{mg} \pm 8.84\text{mg}$) that were more than 18% ($P < 0.05$) lighter than those produced by control ticks (antigen profile 3.5.2.3). There was no significant difference ($P > 0.05$) in engorged tick weight between the UNE group and the nitrocellulose-antigen group.

3.6.6 Immunisation with Whole *Rh. appendiculatus* Unfed Nymphal Extract and UNE-Antilarval Serum Complexes (Guinea Pig and Rabbit Sera).

Table 3.25 shows the effect on tick weight of antigen-antibody complexes. The UNE group gave a reduction in engorged tick weight ($338.90\text{mg} \pm 21.52\text{mg}$) of just over 22.5% ($P < 0.01$) compared to ticks ($438.68\text{mg} \pm 22.05\text{mg}$) from the control group. Inoculation with a UNE-antibody complex prepared using immune rabbit serum (see 2.3.7) only achieved a reduction in tick weight ($389.13\text{mg} \pm 15.00\text{mg}$) of just under 11.5% (not significant, $P < 0.10$), whilst an identical complex prepared using immune guinea pig serum induced a reduction in tick weight ($238.36\text{mg} \pm 28.53\text{mg}$) of just over 45.5% ($P < 0.001$) (antigen profiles 3.5.4). Weight of egg masses were not determined for this experiment. Engorged tick weights from the rabbit antibody complex group were not significantly ($P > 0.05$) different from either the control or UNE groups; but tick weights from the UNE group were significantly different to controls ($P < 0.05$). Engorged ticks from the guinea pig complex group were significantly ($P < 0.05$) lighter than those from the UNE and rabbit complex groups. The tick weight reduction induced by the guinea pig complex when compared to the UNE was 29.7%.

TABLE 3.24 RESULTS OF *RH. APPENDICULATUS* CHALLENGE FEED ON GUINEA PIGS IMMUNISED WITH WHOLE UNFED NYMPHAL EXTRACT AND AN ELECTRO-PHORETICALLY ISOLATED ANTIGEN FRACTION.

MATERIAL INOCULATED	FEMALE ENGORGED WEIGHT (mg) (MEAN ± SE)	REDUCTION IN ENGORGED WEIGHT (%)	WEIGHT OF EGG MASS (mg)	REDUCTION IN EGG MASS WEIGHT (%)
FIA	445.68 ^A ±18.21	-	151.22 ±8.75	-
UNE	303.00 ^B ±27.17	32.01 (P<0.001)	115.62 ±11.75	23.54 (P<0.05)
Nitrocellulose UNE - antigen bearing particles	269.62 ^B ±27.10	39.50 (P<0.001)	123.76 ±8.84	18.16 (P<0.05)

SE Standard Error of Mean FIA Freunds Incomplete Adjuvant
 UNE Unfed Nymphal Extract * Figures with dissimilar superscripts are significantly different (P<0.05)

TABLE 3.25 RESULTS OF *RH. APPENDICULATUS* CHALLENGE FEED ON GUINEA PIGS IMMUNISED WITH WHOLE UNFED NYMPHAL EXTRACT AND UNE-ANTILARVAL SERUM COMPLEXES.

MATERIAL INOCULATED	FEMALE ENGORGED WEIGHT (mg) (MEAN \pm SE)	REDUCTION IN ENGORGED WEIGHT (%)	WEIGHT OF EGG MASS (mg)	REDUCTION IN EGG MASS WEIGHT (%)
FIA	438.68 ^A \pm 22.05	-	-	-
UNE	338.90 ^B \pm 21.52	22.74 (P<0.01)	-	-
UNE-antilarval (guinea pig) serum complex	238.36 ^C \pm 28.53	45.66 (P<0.001)	-	-
UNE-antilarval (rabbit) serum complex	389.13 ^{AB} \pm 15.00	11.30 (P<0.10)	-	-

SE Standard Error of Mean FIA Freund's Incomplete Adjuvant
UNE Unfed Nymphal Extract * Figures with dissimilar superscripts are significantly different (P<0.05)

3.6.7 Immunisation with Whole *Rh. appendiculatus* Unfed Nymphal Extract using Freund's Incomplete Adjuvant or Quil A as an Immunostimulating Agent.

The results of a comparison between the adjuvant, FIA, and an alternative adjuvant, Quil A, are presented in table 3.26. Compared to engorged ticks ($445.68\text{mg} \pm 18.21\text{mg}$) in the control group, UNE mixed with FIA gave a tick weight ($303.00\text{mg} \pm 27.17\text{mg}$) reduction of 32% ($P < 0.001$) and an egg mass weight ($115.62\text{mg} \pm 11.75\text{mg}$ compared to $151.22\text{mg} \pm 8.75\text{mg}$ for the control group) reduction of 23.5% ($P < 0.005$), as against reductions of nearly 69% ($P < 0.001$) (tick weight, $138.75\text{mg} \pm 17.77\text{mg}$) and just under 65% ($P < 0.001$) (egg mass weight, $53.03\text{mg} \pm 8.44\text{mg}$) when UNE was administered with Quil A. UNE immunised with Quil A was found to be significantly better ($P < 0.05$) at reducing engorged tick weight than UNE with FIA. The reduction induced by UNE + Quil A when compared to UNE + FIA was 54.2%. In addition, antibody (IgG) titres were shown to be significantly higher (figure 3.6.7) in guinea pigs immunised with UNE + Quil A (endpoint dilution $1 \times 10^{-6.0}$) than in guinea pigs immunised with UNE + FIA (endpoint dilution $1 \times 10^{-5.41}$).

3.6.8 Immunisation with Whole *Rh. appendiculatus* Unfed Nymphal Extract and Its Membrane Bound Protein Fraction.

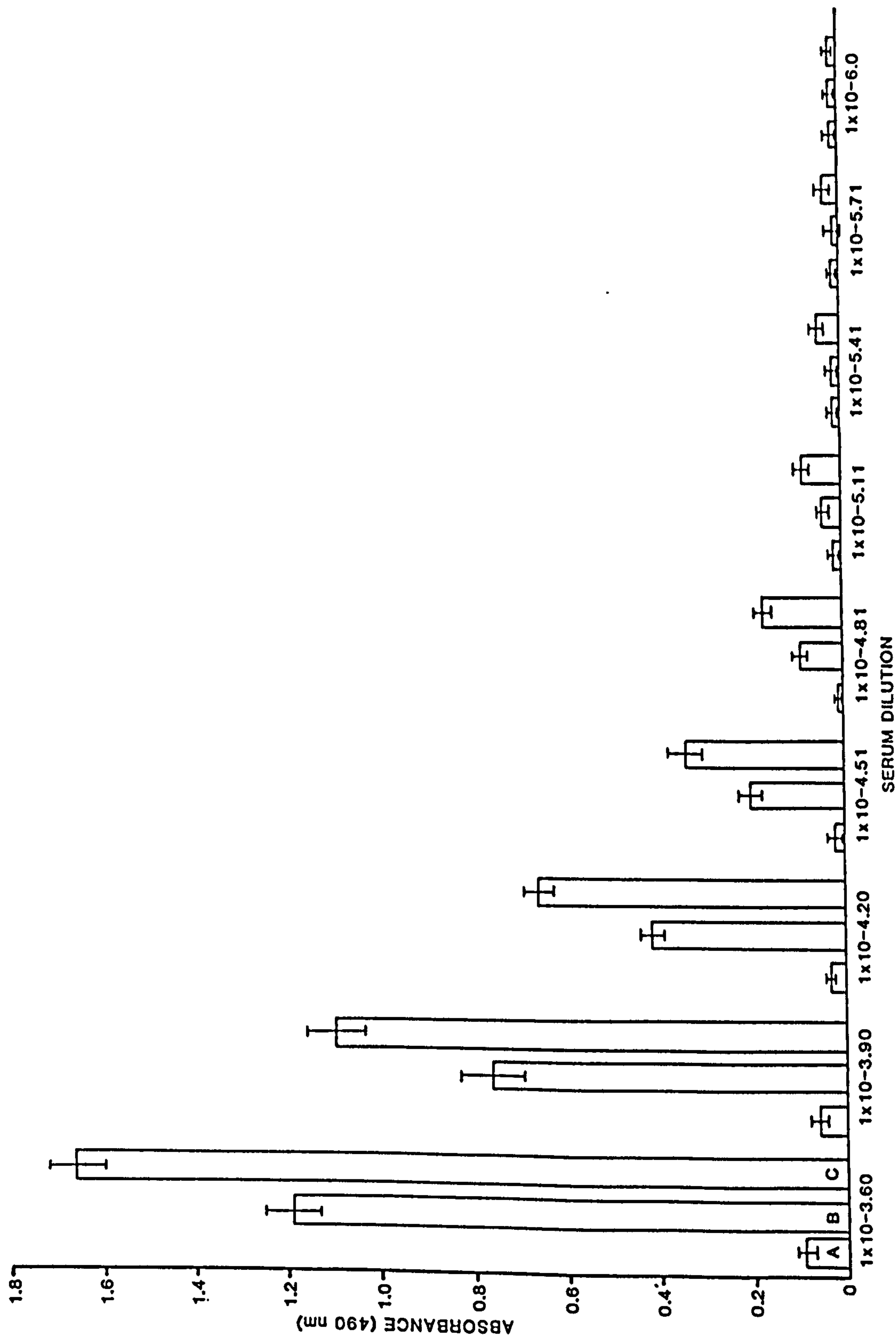
A membrane protein fraction (for antigen profile see 3.5.3) from UNE induced a reduction in engorged tick weight ($305.77\text{mg} \pm 28.24\text{mg}$) of 31.8% ($P < 0.001$) compared to ticks ($448.34\text{mg} \pm$

TABLE 3.26 RESULTS OF *RH. APPENDICULATUS* CHALLENGE FEED ON GUINEA PIGS IMMUNISED WITH WHOLE UNFED NYMPHAL EXTRACT USING FREUNDS INCOMPLETE ADJUVANT OR QUIL A AS AN IMMUNOSTIMULATING AGENT.

MATERIAL INOCULATED	FEMALE ENGORGED WEIGHT (mg) (MEAN ± SE)	REDUCTION IN ENGORGED WEIGHT (%) (P<0.001)	WEIGHT OF EGG MASS (mg) (P<0.001)	REDUCTION IN EGG MASS (%) (P<0.001)
CONTROL (not inoculated)	445.68 ^A ±18.21	-	151.22 ±8.75	-
UNE-FIA	303.00 ^B ±27.17	32.01 (P<0.001)	115.62 ±11.75	23.54 (P<0.05)
UNE-QUIL A	138.75 ^C ±17.77	68.87 (P<0.001)	53.03 ±8.44	64.93 (P<0.001)

SE Standard Error of Mean FIA Freund's Incomplete Adjuvant
UNE Unfed Nymphal Extract * Figures with dissimilar superscripts are significantly different (P<0.05)

Figure 3.6.7 Antibody Responses of Guinea Pigs Immunised with UNE + Quil A and UNE + FIA. Key: A normal guinea pig serum; B serum from guinea pigs immunised with UNE + FIA ; C serum from guinea pigs immunised with UNE + Quil A. UNE unfed nymphal extract; FIA Freunds incomplete adjuvant.



19.99mg) from control guinea pigs (table 3.27); this compared with a figure ($332.97\text{mg} \pm 17.46\text{mg}$) of just over 25.5% ($P < 0.001$) for the UNE. From the data in table 3.27 it can be seen that the difference in tick weight reduction between the unfractionated UNE and its membrane protein fraction was not statistically significant. Weights of egg masses were not determined for this experiment. There was no significant difference ($P > 0.05$) in engorged tick weight between the UNE group and the membrane fraction group.

TABLE 3.27 RESULTS OF *RH. APPENDICULATUS* CHALLENGE FEED ON GUINEA PIGS IMMUNISED WITH WHOLE UNFED NYMPHAL EXTRACT AND ITS MEMBRANE BOUND PROTEIN FRACTION.

MATERIAL INOCULATED	FEMALE ENGORGED WEIGHT (mg) (MEAN ± SE)	REDUCTION IN ENGORGED WEIGHT (%)	WEIGHT OF EGG MASS (mg)	REDUCTION IN EGG MASS WEIGHT (%)
QUIL A (control)	448.34 ^{A*} ±19.99	-	-	-
UNE-QUIL A	332.97 ^B ±17.46	25.73 (P<0.001)	-	-
UNE membrane proteins QUIL A	305.77 ^B ±28.24	31.80 (P<0.001)	-	-

SE Standard Error of Mean FIA Freunds Incomplete Adjuvant
 UNE Unfed Nymphal Extract * Figures with dissimilar superscripts
 are significantly different (P<0.05)

4. DISCUSSION.

4.1 Antigen Identification.

4.1.1 Antigens Involved in Naturally Acquired Immunity.

The antigen profiles of the three different stages of *Rh. appendiculatus* (summarised in table 3.5.1.1), revealed that unfed larval and nymphal extracts and unfed, partially fed or fully fed adult extracts when exposed to sera raised by larval, nymphal or adult challenge feeds, provide positive evidence for the presence of shared interstadial antigens. Such antigens were clearly identified at 70, 55, 51.5, 40, 36.5 and 23kDa in some or all of the extracts by some or all of the sera. Shapiro *et al.* (1986) reported that many of the antigens recognised in extracts of unfed adult *Rh. appendiculatus* by sera from rabbits made immune by adult tick feed were absent in extracts of unfed larval and nymphal instars. This^{is} in contrast to the results I present here. Although there are distinct variations between the different stages, the majority of antigens appear to be present in each stage. However, the difference between adult ticks before, during and after engorgement is more marked. When Shapiro *et al.* (1986) probed extracts with sera from rabbits made resistant by adult tick feed (2 sequential infestations), they observed a greater number of antigens in extracts of salivary glands from unfed adults than they did in extracts of unfed larvae or nymphs. Extracts of whole ticks would be expected to contain many antigens, including those from the salivary glands. I did not

look at salivary gland extract from unfed ticks; but extracts of whole unfed adult ticks had a similar number of antigens as extracts of unfed larvae or nymphs. Shapiro *et al.* list four major antigen bands in unfed immature ticks; this compares with at least seven major bands and several other more weakly stained bands when I probed extracts of unfed immature ticks with antiadult (2 sequential infestations) serum. Furthermore, only one of Shapiro *et al.* antigens (35kDa) fits with the antigen profile of unfed larval extract obtained by myself with antiadult serum (36.5kDa). In the case of unfed nymphal extract, two of Shapiro *et al.* antigens (68 and 35kDa) may be related to antigens identified by myself (70 and 36.5kDa). In contrast to this, Shapiro *et al.* identified five major antigens in extracts of salivary glands from unfed adults together with a further six weaker bands whilst I observed a total of 12 antigenic bands in extracts of salivary glands from partially fed females. Ten of these were considered significant, but only five were strongly labelled. Again, though there is some overlap, marked differences remain; most notably, I detected a strongly antigenic band at 70kDa, but Shapiro *et al.* did not. I observed significant differences in the antigen profiles of fed and unfed ticks, and this would presumably also be the case with their salivary glands. It is important for us not to draw too many conclusions from such comparisons, as differences in methodology and stage of salivary gland may significantly affect the results obtained. For example, Shapiro *et al.* used a first antibody dilution of 1:100, labelled the antibody with iodinated protein A

and visualised antibody binding by autoradiography; whilst I used a first antibody dilution of 1:200, followed by incubation with a peroxidase conjugated second antibody (diluted 1:1000) which was visualised by addition of peroxidase substrate. Another important difference between the two systems is the source of the antiserum. Shapiro *et al.* used rabbits as hosts, whilst I used guinea pigs. When I probed an UNE/guinea pig antilarval tick serum complex with guinea pig antiadult tick serum, bands at 51.5, 36.5, 27 and 23kDa were identified (see table 3.11). These compared with bands of 84, 77.5, 51.5, 36.5 and 23kDa when an UNE/rabbit antilarval tick serum complex was probed with rabbit antiadult tick serum (presumably, the inability of the rabbit complex to induce a significant level of immunity in guinea pigs was due to the recognition of the immunoglobulin part of the complex as being foreign, and any subsequent immune response being directed against the rabbit immunoglobulin instead of, or in addition to, that against the tick antigens). So, it is possible that variations between my results and those of Shapiro *et al.* may be due, at least in part, to the different host species. Interestingly, when Mongi *et al.* (1986b) immuno-precipitated *Rh. appendiculatus* fed female extract with antisera from rabbits exhibiting acquired immunity to adult *Rh. appendiculatus*, no tick antigens were identified.

I have gone further than Shapiro *et al.* (1986) and have compared the profiles of the different stages when probed with antinymphal and antilarval feed sera, in addition to antiadult serum. By analysing extracts of unfed, partially fed and fully fed adults,

the role of the feeding process in the elaboration and the degeneration of antigen species was studied. The 70kDa species identified in each of the three tick stages, and also throughout adult feeding, by antilarval serum was not identified in ULE or UAdE when probed with antinympal or antiadult sera. It is possible that several different antigens (each of similar molecular weight as determined by SDS-PAGE) are recognised in the 70kDa band by the various antisera. In addition the quantity of a particular antigen present in each tick stage will vary, and it is also likely that different reactive epitopes occur in different stages and also perhaps at different points of the feeding cycle. It is also possible that one or more of the reactive epitopes may occur on different proteins in larval, nymphal and adult stages (interference with these epitopes may assist in protecting the host against tick challenge), as has shown to be the case in nematodes (Maizels *et al*, 1987). Two-dimensional electrophoresis would resolve the question of how many different components are present in the band; and the use of monoclonal antibodies would provide us with information about specific epitopes. Neither of these were done in the present studies. The 55kDa antigen was labelled in all three stages by each of the three sera but was found to diminish in females during feeding and disappeared altogether in fully engorged females. Presumably then, this antigen is associated with the early part of the feeding cycle. Two other antigens, at 36.5 and 23kDa also appear to be involved in the early part of the feeding process, having completely disappeared from adult ticks within

six days of the onset of feeding (partially fed female extract) and remaining absent after that (fully fed female extract); and a 40kDa antigen identified by antilarval and antiadult sera, was also found diminished in fed adult ticks. It is likely therefore, that these antigens have a role in the initiation of feeding. Interestingly, despite the 36.5 and 23kDa species being positively labelled by antiadult serum, in extracts of unfed larval or unfed nymphal instars, neither of the bands were identified in extracts of any of the three instars by sera raised to larval and nymphal feeds, suggesting that these two antigen species are not exposed to the host's immune system in the process of feeding by *Rh. appendiculatus* immatures. Alternatively, it may be that the quantities of these antigens involved in the feeding of immature *Rh. appendiculatus* may be too small for the present immunoblotting assay; quantity presumably being related to the size of the feed, the amount of saliva injected and the length of the feeding period (generally shorter for immatures of the species than for adults). Positive labelling of the 36.5 and 23kDa species by antiadult serum (see table 3.3) indicated the presence of highly reactive epitopes; yet sera raised by vaccination with ULE, UNE or UAdE all failed to identify either of these bands (see table 3.4). This suggests that these particular antigens were either degraded upon exposure to the hosts immune system, or that they were not presented in an immunogenic state when vaccinated. Purification to homogeneity of the 23 or 36.5kDa species would permit the development of an ELISA suitable for determining the previous exposure of cattle to adult *Rh. appendiculatus* and possibly, also, their immune status.

A 51.5kDa antigen appeared enhanced by feeding, staining more strongly in extracts of partially fed and fully fed ticks; and a 30kDa species was observed only in extracts of fully fed adults. Whether the 30kDa antigen was a degradation product of a larger molecule or whether it was a new molecule elaborated during the feeding process was unclear. Some of the differences observed in partially and fully fed female extracts when compared to unfed adult extracts may have^{been} due to the additional presence of male ticks in the unfed adult extract. Changes in antigen profiles (salivary glands) due to feeding have, not surprisingly, been observed in several other tick species. Brown (1986) found that a low molecular weight antigen (20kDa) identified in *Am. americanum* salivary gland extract by rabbit antiadult *Am. americanum* serum, was present only at certain stages of the feeding cycle. In contrast, Needham *et al.* (1986) working with the same tick species, observed a 200kDa antigen in salivary gland extract from unfed ticks, which was shown to be absent in extracts of salivary glands from fed ticks. Such extracts from fed ticks were seen to contain two other antigens (165 and 130kDa) which were absent from unfed tick salivary gland extracts. Tracey-Patte (1980) observed that esterases identified in extracts of *Bo. microplus* unfed larvae were found to be absent in extracts of fed larvae, and Gordon and Allen (1987) suggested that the secretion of two antigens into the host soon after the attachment of *De. andersoni* adults might be of importance in tick resistance.

4.1.2 Antigens Involved in Immunity Induced by Vaccination.

From the immunisation/tick challenge feed results obtained in section 3.6, it is evident that both the 45% ammonium sulphate supernate fractions and the SEHPLC fraction 2 of unfed nymphal extracts were good sources of protective antigen(s). In all experiments involving either of these two fractions, the levels of immunity to *Rh. appendiculatus* adults exhibited by guinea pigs immunised with one of these fractions were significantly superior ($P < 0.05$) to those exhibited by any other fractions and were always as good as ($P < 0.05$) or better than the unfractionated extract. As a consequence, antisera raised against these fractions were used to identify antigens present in or absent from all fractionated extracts.

From the antigen profiles obtained in section 3.5 by immunostaining extract fractions with anti45supt serum, antiF2 serum or their own post immunisation serum, and from the immunisation/tick challenge experiments in 3.6, it can be seen that in the majority of protective fractions, antigenic components were recognised at 97.5, 93.5, 84, 70, 60 and 40kDa (summarised in tables 4.1 and 4.2). Of these bands, the 84, 70 and 60kDa species each stimulated antibody production in all successful immunisations, except for immunisation with the guinea pig immune complex; and then, even though the complex antiserum did not recognise any of the three bands, the 60kDa species was identified in the complex when probed with anti45supt serum.

TABLE 4.1 SUMMARY OF MAJOR ANTIGENS IDENTIFIED IN FRACTIONS OF RH. APPENDICULATUS UNFED LARVAL AND NYMPHAL EXTRACTS BY POST IMMUNISATION (45% AMMONIUM SULPHATE SUPERNATE FRACTION AND SEHPLC FRACTION 2 OF UNE) SERA.

ANTISERUM	ANTIGEN SOURCE	ANTIGEN MOLECULAR WEIGHT (kDa)					
		97.5	93.5	84	70	60	40
Anti45supt	ULE, charcoal treated	+	+	+/-	-	+	+
	" , dialysed	+	+	+/-	-	+	+
	" , PEG 8000 supernate	+	+	+/-	-	+	+/-
	" , ammonium sulphate precipitate	+	+	-	-	+	-
	" , ammonium sulphate supernate	+	+	+/-	-	+	+
AntiF2	UNE, SEHPLC fraction 1	+	+	+	+	+/-	-
	" , " " 2	+	+	+	+	+	+
	" , " " 3	+	+	+/-	+	-	+
Anti45supt	" , " " 2	+	+	+/-	+	+	+
AntiF2	UNE, SEHPLC fraction 1a	+	+	+	+	-	-
	" , " " 2a	+	+	+	+	-	+/-
	" , " " 3a	+	+	+/-	+	+/-	+/-
Anti45supt	" , " " 2a	+	-	+	+	+	+
	" , " " 3a	+	-	-	+	+	+
Anti45supt	UNE, 20% ammonium sulphate precipitate	+	+	+	+	+	+
	" , 35% ammonium sulphate precipitate	+	+	+	+	+	+
	" , 45% ammonium sulphate precipitate	+	+	+	+	+	+
	" , 45% ammonium sulphate supernate	+	+	+/-	+	+	+
AntiF2	UNE, membrane fraction	+	+	-	+	-	-
	" , soluble "	+	+	+	+	-	-
Anti45supt	" , membrane "	+	+	-	-	+	-
	" , soluble "	+	+	+	+	+	+
Anti45supt	UNE-guinea pig antilarval serum complex	+	+	-	-	+	-

+ Labelled - Unlabelled ULE Unfed Larval Extract UNE Unfed Nymphal Extract

TABLE 4.2 SUMMARY OF MAJOR ANTIGENS IDENTIFIED IN RH. APPENDICULATUS UNFED LARVAL AND NYMPHAL EXTRACTS AND THEIR FRACTIONS BY THEIR POST IMMUNISATION SERA.

ANTISERUM TO	ANTIGEN MOLECULAR WEIGHT (kDa)					
	97.5	93.5	84	70	60	40
ULE	+	+	+	+	+	-
UNE	+	+	+	+	+	-
UNE, SEHPLC fraction 1	+	+	-	+	-	-
" , " " 2	+	+	+	+	+	+
" , " " 3	+/-	+	-	+	-	+/-
UNE, 45% ammonium sulphate supernate	+/-	+	+/-	+	+	+
" , 45% ammonium sulphate supernate (135µg)	-	-	+	+	+	+
" , 45% ammonium sulphate supernate (1350µg)	+/-	+	+	+	+	+
UNE, NCP antigens	+	+	+/-	+	+	+
UNE-guinea pig antilarval serum complex	+	+	-	-	-	-

+ Labelled - Unlabelled ULE Unfed Larval Extract UNE Unfed Nymphal Extract NCP Nitrocellulose Particle

Fractions 1 and 3 of UNE induced levels of protection in guinea pigs to adult tick challenge that were markedly less than that induced by fraction 2 (see table 3.19). The 84 and 60 kDa antigens both identified in fraction 2 by its own antiserum, were not identified in fractions 1 and 3 by their own antisera. The 40kDa antigen followed a similar trend, though it was present as a minor component in fraction 3. Fraction 2a of UNE when probed with antiF2 serum did not exhibit a 60kDa antigen species, whilst fraction 3a did. The 84 and 40kDa antigens were more strongly identified in fraction 2a than in fraction 3a, but it was fraction 3a that induced a greater level of immunity in guinea pigs, as expressed by reduction in engorged tick weight when compared to controls; thus further indicating that the 60kDa species may be important in the development of immunity to *Rh. appendiculatus*.

In one immunisation experiment the reduction in engorged tick weight was enhanced by the elimination (by diluting out) of several antigen species. Only the 84, 70, 60 and 40kDa species were identified when a 45% ammonium sulphate supernate from an unfed nymphal extract was probed with antiserum raised against a reduced dose of the same supernate. Additionally, in several cases (eg. 45% ammonium sulphate precipitate) the antigens were identified in extract fractions, but there was little or a reduced level of immunity brought about by immunising animals with such fractions. It is quite likely that the immune response following exposure to these antigens was suppressed by other components in the extract. Such antigenic competition has been

reported elsewhere (Hammerl *et al*, 1988), and it has been suggested that different antigenic competition effects are induced by different protein compositions of antigenic mixtures, and that such competition may determine the immunogenicity of certain molecules quite apart from the immunogenic properties of the molecules themselves. Comparing my results with those of Mongi *et al*. (1986b) proves confusing. Mongi *et al*. analysed immune complexes (formed by immunoprecipitation of fully fed female extract with serum from rabbits immunised by inoculation) using SDS-PAGE. Bands on gels were identified as being between 82 and 180kDa. In comparison, many of the antigens identified in blotting studies carried out by myself, were below 82kDa in molecular weight, and in one successful immunisation (see tables 3.9 and 3.23) apart from an 84kDa species no antigens above 70kDa were observed. Shapiro *et al*. (1987) detected a 90kDa antigen, together with one of 300kDa upon immunoblot analysis of a salivary gland extract with serum raised against a precipitin arc obtained by crossed immunoelectrophoresis. The 90kDa species could well be similar to one of the antigens identified by myself. As can be seen from the immunoblots in 3.5.1.1 and 3.5.1.2, the staining of the 97.5 and 93.5kDa bands is very strong, and this may be due to overlapping of different antigen species. In addition to the 90kDa antigen of Shapiro *et al*, which reduced tick feeding, two other precipitin arcs were used to immunise rabbits, but both of these antigens appeared to enhance tick feeding. These components were not analysed by immunoblotting and consequently their molecular weights are not available.

Despite no recorded evidence of cross-immunity between *Rh. appendiculatus* and other tick species following infestation, when unfed larval extracts of various species (including *Am. hebraeum* and *Am. variegatum*, two of the tick species that overlap in distribution with *Rh. appendiculatus*) were probed with serum raised by immunisation with material fractionated from *Rh. appendiculatus* unfed nymphal extract (anti45supt) several antigens of similar molecular weight were observed in the different tick species (see table 3.12). Interestingly only antigens of 60kDa were identified in all five species of tick, and 60kDa species were the only antigens common to *Rh. appendiculatus* and the two *Amblyomma* species. Such an observation is of particular interest as this molecular weight also represents one of the antigens most likely to induce some form of immunity to *Rh. appendiculatus*, and suggests that some degree of cross-protective immunity between *Rh. appendiculatus* and both *Am. hebraeum* and *Am. variegatum* immatures may be exhibited in animals vaccinated with ULE of these species. There is some evidence for this, Heller-Haupt and Varma vaccinated guinea pigs with an extract of *Am. variegatum* unfed larvae and challenged the immunised guinea pigs with *Rh. appendiculatus* larvae; engorged weight of the fed larvae was found to be slightly but significantly reduced, indicating that vaccination induced some cross-protective immunity (Heller-Haupt and Varma, personal communication).

Mongi *et al.* (1986b) and Shapiro *et al.* (1986 and 1987) used rabbits in their experimental systems, and I used guinea pigs in my work. Just how relevant to field conditions all these results are is questionable. Recent successful immunisation of sheep against *Rh. appendiculatus* by inoculation with a 45% ammonium sulphate supernate fraction of an unfed nymphal extract suggests that the antigenicity of the fraction is similar in sheep and guinea pigs (Subandrio, 1988). Intradermal inoculation of the 45% ammonium sulphate supernate in Zambian 'Tonga' cattle induced a delayed type hypersensitivity response in tick-resistant cattle, but not in tick-susceptible cattle. This suggests that the fraction is potentially immunogenic in cattle as well as in sheep and guinea pigs (Smith *et al.*, in Press).

4.2 Biochemical Characterisation of Antigens.

Of the characterisation studies carried out on tick proteins most have involved the identification of certain types of proteins such as esterases (Geczy *et al.*, 1971; Willadsen and Williams, 1976 and Reich and Zorzopulos, 1980), proteases (Willadsen and Riding, 1979), acid and alkaline phosphatases (Tatchell, 1971 and Gill *et al.*, 1986) and aminopeptidases (Gill *et al.*, 1986).

Both Willadsen *et al.* (1988) and Opdebeeck *et al.* (1988a) have indicated that the antigen(s) associated with immunity to *Bo. microplus* is/are membrane associated. My results indicate that this may also be the case with some of the reactive antigens in

Rh. appendiculatus. However, several antigens in this species do not appear to be membrane associated (eg. 84, 77.5, 75 and 40kDa), and those that are, also appear in soluble form. The 70kDa antigen was positively identified in the membrane fraction by the antiF2 serum, but not by the anti45supt serum; whilst the 60kDa species was recognised in the membrane fraction by the anti45supt serum, but not by the antiF2 serum. It is possible that the two sera each recognise different epitopes on the molecules, and that the harsh detergent extraction (Nonidet NP-40) denatured some of the epitopes. Alternatively, perhaps membrane association involves a conformational change which makes some epitopes more accessible and other epitopes less accessible than when the molecule is in the soluble phase.

Gill *et al.* (1986) identified several antigens in extracts of *Hy. anatolicum anatolicum* salivary glands as being glycoprotein in nature. Twelve out of 17 antigens identified bound the lectin concanavalin A. I assayed extracts of *Rh. appendiculatus* unfed nymphs for a range of carbohydrates and found that most of the proteins present were heavily glycosylated, binding several different lectins displaying a range of carbohydrate specificities. Obviously, although the degree of binding is related to the specific carbohydrate content of a particular antigen, it is also affected by the amount of antigen present; so although the 97.5 and 93.5kDa antigens appeared most heavily glycosylated, it may be that they are just particularly abundant. The high affinity of the lectins for many of the proteins indicates the presence of methyl glucoside and oligosaccharide

side chains on the protein molecules, rather than just simple sugars. Of the major antigens identified, the 84, 70 and 40kDa species were all clearly glycosylated, exhibiting a broad range of carbohydrate moieties; whilst the 60kDa species appeared only very weakly glycosylated. Both the 70 and 40kDa antigens were often identified in immunoblotting studies as broad, diffuse bands; this suggests heterogeneity. Presumably, as these antigens are both glycosylated the heterogeneity is due to variations in the oligosaccharide side chain(s), for example in the sialic acid content. If any of the reactive epitopes are on the carbohydrate moiety of the antigen, then such heterogeneity would explain the observed variations in the binding of antibody together with variations in the degree of protection induced by a particular antigen. Cleavage of the carbohydrate side chain(s) and subsequent analysis of the residual protein by immunoblotting would indicate whether the epitopes are on the carbohydrate and/or the protein part of the molecule. For example, terminal sialic acids can be removed using neuraminidase (Shimohigashi and Chen, 1985). Such knowledge would be of major importance if a particular antigen is to be manufactured in large quantities using recombinant DNA technology (see section 4.5).

The molecular weight range observed in HPLC size-exclusion studies was much greater than that seen in studies by electrophoresis. SEHPLC fraction 1 for example contained material ranging from nearly 700kDa molecular weight downwards (as determined by SEHPLC, under non-reducing conditions, section

3.3), very little of this material was below about 120kDa. When the same fraction was analysed by SDS-polyacrylamide gel electrophoresis under reducing conditions (section 3.4) most of the proteins/polypeptides were observed to be between 50 and 100kDa. This suggests that at least several of the proteins exist in their native state as polymeric structures, either instead of or in addition to their monomeric form.

4.3 Localisation of Antigen in Tick Tissues.

Agbede and Kemp (1986), Willadsen and Kemp (1988) and Opdebeeck *et al.* (1988b) have shown that the antigen(s) associated with vaccination induced immunity to *Bo. microplus* is/are clearly associated with the gut cell membranes of the adult female tick. From table 3.5.7, it can be seen that in *Rh. appendiculatus* this does not appear to be the case. The only recognition of gut associated antigen(s) by antibody was with serum raised against a whole unpurified tick extract (unfed nymphal extract). However, several points should be noted: *Bo. microplus* is a one host tick and remains on the host throughout maturation from larva through adult; whilst *Rh. appendiculatus* is a three host tick and detaches after each engorgement to moult. Additionally, the tick stage of the extract used for inoculation is different (partially fed adults in the case of *Bo. microplus* and *Rh. appendiculatus* unfed nymphs in my work).

When serum against the 90kDa antigen identified by Shapiro *et al.* (1987) was used as the antibody source in immunocytochemical labelling of *Rh. appendiculatus* salivary glands, the glands' complex a-, d- and e-granules were labelled together with the walls of intercalated ducts in feeding adults. Additionally, labelled e-granules were also identified within the labyrinthine spaces of the salivary gland type III acini (Venable *et al.*, 1986). In studies done in this laboratory, and presented here (Bechara, personal communication), sections of whole unfed *Rh. appendiculatus* females were labelled using sera raised against whole and fractionated extracts of unfed *Rh. appendiculatus* nymphs and also against hyperimmune serum raised by adult challenge feed. As expected, the antiadult serum heavily labelled antigens associated with all three types of salivary gland acini; and not surprisingly so too were the salivary ducts, the oesophageal tract and lumen of the malphigian tubules, but not the gut. In comparison, all of the vaccination antisera labelled antigenic material associated with the salivary gland type I acini, but apart from the antiUNE serum (lumen of acini types II and III) and the anti45supt serum (weakly in lumen of type II acini) the type II and type III acini were unlabelled. This suggests that the antigen(s) involved in vaccination induced immunity in my experiments, differ(s) from the antigen identified by Shapiro *et al.* (1987). This is further substantiated by the earlier observation that antiserum to the low dose 45% ammonium sulphate supernate did not recognise any material above 84kDa in molecular weight (sections 3.5.2.3 and 4.1.2). Of the various tissue types labelled by vaccination antisera, the haemocytes and

haemolymph were labelled to the greatest degree. Notably, only vaccination with nitrocellulose antigen-bearing particles failed to raise antibodies that recognised antigen associated with the haemocytes, but in this case the haemolymph was very strongly labelled. Perhaps this was due to a conformational change in the antigen structure due to the harsh denaturation procedure involved in the electrophoretic separation of material associated with the preparation of the antigen-bearing particles (boiling in mercaptoethanol and SDS). Walker *et al.* (1985) have suggested that the salivary gland type I acini have an osmoregulatory function. Such a role must involve a close inter^rrelationship with the haemolymph, and could explain why the type I acini were consistently labelled along with the haemolymph and the haemocytes.

Several authors have suggested that along with salivary glands and gut, tick haemolymph may be a source of antigens targeted by the host (Roberts, 1968; Galun, 1978; Ackerman *et al.*, 1980; McGowan *et al.*, 1980; Binta *et al.*, 1985 and Ben Yakir and Barker, 1987), and there are indications that haemolymph proteins may be transported to the salivary glands (Binnington and Kemp, 1980). The haemolymph serves as a transport system for nutrients, hormones, metabolic intermediates and specific pathogens to various internal organs of the tick (Neitz and Vermeulen, 1987). Tissues of internal organs such as salivary gland alveoli, ovarian oocytes, fat body, malpighian tubules and midgut epithelium are separated from the surrounding haemolymph by only a thin basement membrane (Binnington and Obenchain, 1982); so

haemolymph affects both the biochemical characteristics of the entire tick and also its vector potential. There has been little done in the way of identifying proteins present in tick haemolymph. Various enzymes (esterases, acid and alkaline phosphatases, leucine aminopeptidase) have been identified in *Bo. microplus* haemolymph (Tatchell, 1971), as have haemoglycoproteins (Tatchell, 1971); lipovitellins also have been identified in the haemolymph of *De. andersoni* (Boctor and Kamel, 1976). Haemolymph proteins will be influenced by the developmental stage, physiological state and sex of the ticks, and possibly also by the host (Dolp and Hamdy, 1971). However, certain proteins do appear to be common to both nymphs and adults (Dolp and Hamdy, 1971) and this would presumably be the case with larvae also; so immunisation with female haemolymph might be thought to induce immunity in more than one tick stage.

Nogge and Giannetti ((1980) fed tsetse flies on a diet containing antiserum specific for an osmoregulatory protein in their haemolymph, as a result of which all the flies died; but when Ben Yakir and Barker (1987) immunised rabbits with cell free haemolymph from replete ticks they failed to induce any significant degree of immunity. Perhaps the use of haemolymph from partially fed or unfed (difficult to obtain) ticks would have produced different results, or perhaps haemocyte associated antigens are required in addition to haemolymph antigens. Work in this laboratory has centred around the use of whole tick extracts, which probably contain antigens from several tissues including haemolymph.

4.4 Variability of the Immune Response.

Johnston *et al.* (1986) and Willadsen *et al.* (1988) both reported large variations in the immune response against *Bo. microplus* expressed by individual cattle following vaccination with extracts of partially fed female *Bo. microplus*. Large variations in the levels of immunity induced in individual animals (guinea pigs) by vaccination were also observed in this laboratory. In one experiment, three guinea pigs were each inoculated with a SEHPLC fraction 2 from an unfed nymphal extract (table 3.6.3b). The average reduction in engorged tick weight compared to ticks from control animals was 64.71%, but ticks from one individual guinea pig were reduced in weight by only 49.7%, whilst ticks from both the other guinea pigs were in excess of 70% lighter. One of these two guinea pigs (75.6% reduction in engorged tick weight) was used as the source of antiF2 serum used in the immunochemical studies in section 3.5. In another experiment (table 3.6.4a) reductions in engorged tick weight, after feeding on animals immunised with a 45% ammonium sulphate supernate fraction of an unfed nymphal extract, ranged from 30.4% with one guinea pig to nearly 70% with another. Serum from a guinea pig on which engorged ticks were 68.6% lighter than those from control guinea pigs was used in section 3.5. Ticks recovered from guinea pigs inoculated with the low dose 45% ammonium sulphate supernate (table 3.6.4c) were, on average, 55.23% lighter than ticks from control animals. Reductions in tick

weight ranged from 26.1% on one animal to nearly 75% on another. A 64.8% engorged tick weight reduction was observed in the guinea pig from which antiserum was removed for use in section 3.5. Johnston *et al.* (1986) suggested that as the mixture of tick antigens becomes less and less contaminated with irrelevant proteins the animal to animal variation will be smaller. Whilst this may in part be true, I feel that this may explain differences between separate identical experiments rather than between individuals within the same experiment. For example, in the results presented here, when guinea pigs were inoculated with whole unfed nymphal extracts, the average engorged tick weight reduction compared to controls ranged from 22.7% to 68.9%. Differences within a particular experiment are more likely due to differences in the immune responsiveness of the individual animals. Whether or not such differences are related to the individual's antibody titres has not been determined.

4.5 Antigen Production and Prospects for the Development of an Anti-*Rh. appendiculatus* Vaccine.

The production of an anti-*Rh. appendiculatus* vaccine does appear to be a feasible objective assuming that is, that the method chosen for antigen screening (immunoblotting) does indeed identify relevant antigen(s) involved in host immunity to the tick. There is strong evidence for a humoral component (alongside cell-mediated reactions) in the host's immune response to ticks (see section 1.12), therefore there is little reason to doubt the relevance or validity of the immunoblotting assay.

Having identified several potential antigens, the obvious next step is to purify the most likely vaccine candidates (84, 60 and 40kDa) to homogeneity and test them in vaccination/tick challenge feed experiments. A sequential purification strategy would probably involve a crude ammonium sulphate precipitation as a first step. Looking at table 3.9, most of the 60kDa antigen is in the 45% precipitate and supernate; therefore preparation of a 35% to 60% w/v (55% to 85% saturation) ammonium sulphate fraction should be suitable. Such a fraction should also contain most of the 84 and 40kDa antigens. Because of the heterogeneous nature of the 40kDa species (see section 4.3), ion-exchange chromatography may be difficult to use as the antigen will be separated over a range of ion concentrations (though not reported here, results of a preliminary ion-exchange fractionation have shown this to be the case); so it will be easier to make use of the lectin binding characteristics of this antigen and pass the desalted ammonium sulphate fraction through a lectin affinity column. The 60kDa species does not appear strongly glycosylated and separation of the ammonium sulphate fraction via an ion-exchange column should allow a significant degree of purification. Either of these methods could be used with the 84kDa antigen which does not appear heterogeneous. The eluate fraction(s) containing the desired (84, 60 or 40Kda) antigen would then be passed through a size exclusion column, antigen containing fractions identified and if necessary purified further using preparative isoelectric focussing and perhaps also

immunoaffinity chromatography. At each stage the fractions would need to be assayed both for the antigen (immunoblotting) and for any contaminating proteins/polypeptides (SDS-PAGE). Initial screening of extracts from various tick tissues might allow the use of the isolated tissue as an antigen source (as has been done with salivary gland and gut) thereby immediately reducing the number of irrelevant antigens. However, from the data presented here it appears that the 84 and 40kDa species are present mainly in unfed *Rh. appendiculatus* ticks, which are difficult to dissect. The 40kDa antigen was not identified in extracts of partially fed or fully fed *Rh. appendiculatus* adults.

Assuming homogeneity, then the pure antigen could be used in a vaccine trial, and assuming a good degree of immunity developed then, in the case of the 60kDa antigen, the resulting antisera could be used to screen a cDNA expression library to *Rh. appendiculatus* for the gene expressing the antigen. Isolation of the gene, insertion into a suitable vector and amplification of the gene would permit large quantities of the antigen to be produced. A strong vector candidate must be vaccinia virus (cow pox). Insertion of foreign genes into the virus has been achieved without loss of viral infectivity and the proteins produced may be secreted or inserted into the viral membrane, as desired (Ada and Jones, 1987; Mackett, 1987; Murray, 1987). Cattle are the main hosts requiring protection from *Rh. appendiculatus* infestation, and this fact would make vaccinia virus a particularly suitable vector. A gene coding for a rabies virus membrane protein has been incorporated into live vaccinia

virus and successfully used for oral vaccination of foxes (Blancon *et al*, 1986). One major advantage is the large size of the vaccinia virus genome, which would permit incorporation of several genes simultaneously, thus permitting the development of a multivalent vaccine (Smith and Moss, 1983). This would be useful if more than one antigen is required to stimulate immunity, or if we wished to vaccinate simultaneously against several tick species.

Immunity to many parasites involves T cell activation, and it is sometimes the case that purified or recombinant antigens induce neutralising antibody (B cells), but fail to induce much in the way of T cell responses (Murray, 1987). This may be due to T cell epitopes on the antigen being denatured or absent; therefore, assay of the purified or recombinant *Rh. appendiculatus* antigen for its ability to stimulate T cell proliferation *in vitro* may assist in further determining the potential immunogenicity of the antigen. If the recombinant antigen was unable to stimulate T cells or failed to induce immunity then it may be possible to use T cell stimulating synthetic peptides along with the antigen as a vaccine.

Identification of the specific peptides making up T and B cell specific peptides on the antigen would be a prerequisite of a synthetic vaccine (Murray, 1987). This would require knowledge of the antigen's peptide sequence together with the spatial conformation of the molecule (Arnon, 1984). Monoclonal

antibodies can be used to assist in identification of B cell epitopes, but locating potential T cell epitopes is not so easy. Various authors have made predictions regarding the conformation of T cell epitopes: De Lisi and Berzofsky have predicted that T cell epitopes consist of peptides forming alpha-helical-amphipathic structures (De Lisi and Berzofsky, 1985; Sponge *et al*, 1987) whilst Rothbard (1986) has suggested that T cell sites contain a charged residue adjacent to two polar residues which in turn are adjacent to a charged or polar residue. Assuming that the relevant peptides could be found then it would be a comparatively easy task to chemically synthesise or genetically engineer a peptide vaccine.

In section 4.3 I mentioned the importance of knowing whether the protective epitopes are on the carbohydrate side chain(s) and/or on the protein part of glycosylated antigens such as the 84 and 40kDa species. If the latter is true, and none of the relevant epitopes are associated with the carbohydrate moiety, then recombinant DNA technology may be used to produce the protein part of the antigen. However, if any of the epitopes are on the carbohydrate side chain(s) then it will prove difficult, if not impossible, to manufacture the complete antigen, as each different carbohydrate will be coded for by a different gene, and further additional genes would be required for assembly molecules. Such a problem has been encountered by Willadsen, Kemp and co-workers, who have cloned an 89kDa protein from *Bo. microplus* partially fed females (World Patent 8803 929, June 1988: Biotechnology Australia; CSIRO). They obtained only about

two thirds of the expected level of immunity when they vaccinated calves with the recombinant protein (Kemp, personal communication). The patent indicates that the protein is glycosylated and, if one assumes that some of the epitopes are on the carbohydrate moiety, this would account for the discrepancy between the immune response to the recombinant protein and that of the purified tick antigen. Obviously, much depends on the extent to which the important epitopes are associated with the carbohydrate moiety of the antigen but if, in the cases of the *Rh. appendiculatus* 84 and 40kDa antigens, genetic engineering did not prove feasible, or if the purified or recombinant antigen failed to stimulate T cells and/or failed to induce a significant degree of immunity to *Rh. appendiculatus*, then the manipulation of antiidiotypic antibodies may be an alternative.

An immune response should involve the formation of antibodies that recognise the idiotypes of antibodies formed against the original antigen. Of these antiidiotypic antibodies, some should recognise the paratope (antigen binding site) of the first antibody and will be essentially an 'internal image' of the original antigen epitope. Antiidiotypic antibodies against both polyclonal and monoclonal antibodies have been successfully used in several systems to induce immunity (Ada and Jones, 1987; Murray, 1987). The first effective antiidiotypic vaccine was against trypanosomiasis (Sacks *et al*, 1982), but it is the successful development of an antiidiotypic vaccine that mimics the epitopes on the carbohydrate moiety of a 38kDa glycoprotein

from *Schistosoma mansoni* that evokes most attention. The vaccine was tested in the rat model of *S. mansoni* and an immunity at least as strong as that induced by any published alternative vaccination methods was seen to develop (Grzych *et al*, 1985). In the case of the *Rh. appendiculatus* 40kDa antigen, monoclonal antibodies could be raised in mice against the purified antigen and used to generate antiidiotypic antibodies in the host (cattle in the field; guinea pigs or rabbits in the laboratory). These could be made idiotypic-specific by adsorption to normal mouse immunoglobulins coupled to an affinity column and the serum IgG used as a surrogate antigen.

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APPENDICES.

Appendix 1: BUFFER RECIPES.

1.A PBS pH 7.3 (Phosphate Buffered Saline, Dulbecco's A).

Made from prepared tablets (Oxoid), 1 tablet in 100ml deionised water. Dissolved tablets constitute:

NaCl	8g.l^{-1}
Na_2HPO_4	1.15g.l^{-1}
KCl	0.2g.l^{-1}
KH_2PO_4	0.2g.l^{-1}

All reagents were equivalent to BDH AnalaR grade (Oxoid data sheet). PBS was sterilised by autoclaving at 115°C for 10min.

1.B HPLC-PBS.

This was made up as in 1.A, but ultrapure water (Milli Q, Millipore) was used instead of deionised water. The PBS was then filtered through a $0.22\mu\text{m}$ durapore membrane using a Millipore negative pressure filter unit, and thoroughly degassed under vacuum.

1.C Saturated Ammonium Sulphate Solution

100g of crystalline ammonium sulphate was dissolved in ^{100ml} distilled water at 50°C and left to stand at room temperature overnight. The supernatant was assumed to be 100% saturated.

1.D Ammonium Sulphate Weight by Volume (w/v) solutions and percent saturation.

The relationship between w/v concentrations of ammonium sulphate and percent saturation is given in the table below.

From S ₁ % \ To S ₂ %	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
0	27	55	84	113	144	176	208	242	277	314	351	390	430	472	516	561	608	657	708	761
5		27	56	85	115	146	179	212	246	282	319	357	397	439	481	526	572	621	671	723
	10		28	57	86	117	149	182	216	251	287	325	364	405	447	491	537	584	634	685
		15		28	58	88	119	151	185	219	255	292	331	371	413	456	501	548	596	647
			20		29	59	89	121	154	188	223	260	298	337	378	421	465	511	559	609
				25		29	60	91	123	157	191	227	265	304	344	386	429	475	522	571
					30		30	61	92	126	160	195	232	270	309	351	393	438	485	533
						35		30	62	94	128	163	199	236	275	316	358	402	447	495
							40	30	62	94	128	163	199	236	275	316	358	402	447	495
								45	31	63	96	130	166	202	241	281	322	365	410	457
									31	63	96	130	166	202	241	281	322	365	410	457
										31	64	97	132	169	206	245	286	329	373	419
									50		32	65	99	135	172	210	250	292	335	381
										55		33	66	101	138	175	215	256	298	343
											60		33	67	103	140	179	219	261	305
												65		34	69	105	143	183	224	266
													70		34	70	107	146	186	228
														75		35	72	110	149	190
															80		36	73	112	152
																85		37	75	114
																	90		37	76
																		95		38

1.E Electrophoresis buffers.

Electrophoresis Running Buffer:

0.025M TRIS (TRIZMA base, Sigma)

0.192M Glycine (Sigma)

0.1% SDS (BDH, Electran grade)

adjusted to pH 8.3

Homogenous 12% Polyacrylamide Gels.

Stacking Gel Buffer:

TRIS, adjusted to pH 6.8 using HCl (BDH, AnalaR)
and diluted to 0.125M

0.1% SDS

Separating Gel Buffer:

TRIS adjusted to pH 8.8 using HCL and diluted
to 0.375M

0.1% SDS

Separating Gel:

9.4g Acrylamide (BDH, Electran)

2.6g N,N'-methylene bisacrylamide (Bio-Rad)

Separating Gel Buffer to 100ml

0.025ml TEMED

0.025g Ammonium persulphate

Stacking Gel:

3g Acrylamide

0.080g N,N'-methylene bisacrylamide

Stacking Gel Buffer to 100ml

0.025ml TEMED

0.025g Ammonium persulphate

Gradient Gels (5% to 20% Polyacrylamide).

Stacking Gel Buffer:

0.6M TRIS adusted to pH 6.8 with HCL

Separating Gel Buffer:

1.875M TRIS adjusted to pH 8.8 with HCl

Stock Acrylamide (30% Acrylamide, 0.8% N,N'-methylene bisacrylamide:

75g Acrylamide

2.0g N,N'-methylene bisacrylamide

distilled water to final volume 150ml

Separating Gel:

	5% Acrylamide (light)	20% Acrylamide (heavy)
TRIS, pH 8.8	3.0ml	3.0ml
distilled water	9.3ml	0.6ml
Stock Acrylamide	2.5ml	10.0ml

10% SDS	0.15ml	0.15ml
5% Ammonium persulphate	0.05ml	0.05ml
Sucrose	-	2.2ml

Stacking Gel:

TRIS pH6.8	1.0ml
Stock Acrylamide	1.35ml
distilled water	7.5ml
10% SDS	0.1ml
5% Ammonium persulphate	0.05ml

1.F Buffers for Western blotting.

Transfer Buffer:

1.25mM	TRIS (TRIZMA base, Sigma)	(3.05g.l ⁻¹)
192mM	Glycine (Sigma)	(14.41g.l ⁻¹)
20% (v/v)	Methyl alcohol (BDH GPR)	(200ml.l ⁻¹)

PBST buffer, pH 7.6:

145mM	NaCl (BDH AnalaR)	(8.5g.l ⁻¹)
9mM	Na ₂ HPO ₄	(1.28g.l ⁻¹)
1mM	NaH ₂ PO ₄ .2H ₂ O	(0.156g.l ⁻¹)
0.3% (v/v)	TWEEN 20 (Sigma)	(3ml.l ⁻¹)

1.G Peroxidase Substrate Buffer

0.3g.l⁻¹ 3,3-diaminobenzidine tetrahydrochloride (Sigma)
in sterile PBS pH7.3 (Appendix 1.A)

60ul 6% H₂O₂

1.H Buffers for Immunostaining of Sections

TBS (TRIS Buffered Saline):

8.1g NaCl
0.6g TRIS (TRIZMA base, Sigma)
3.8ml 1N HCl
Distilled Water to 1000ml

Alkaline Phosphatase Substrate Solution:

2mg Naphthol AS-MX-phosphate (Sigma)
0.2ml Dimethylformamide (Sigma)
9.8ml 0.1M TRIS pH 8.2
10mg Fast Red TR Salt ($1\text{mg}\cdot\text{ml}^{-1}$) was added immediately before use and the solution filtered directly onto sections.

1.I ELISA Buffers

Wash Buffer:

2% Bovine Serum Albumin (Sigma)
0.3% Tween 20 (Sigma)
in PBS pH 7.3 (Appendix 1.A)

Peroxidase Substrate Solution:

0.73g Na_2HPO_4
0.47g Citric Acid
40mg o-Phenylenediamine dihydrochloride (Sigma)
120^ul 6% H_2O_2
Distilled Water to 100ml

Appendix 2: STAINING OF PROTEINS

2.A Visualisation of Proteins in SDS-Polyacrylamide Gels:

Gels were fixed in 20% methyl alcohol/7.5% acetic acid for 1hr prior to staining.

Fast Stain: Coomassie Brilliant Blue G-250 (Sigma)

Stock solution of 2.0g.l^{-1} CBBG-250 in 50% methyl alcohol.
For working solution, 10ml acetic acid (BDH GPR) added to 90ml stock solution.

Stained for 10min.

Slow Stain: Coomassie Brilliant Blue R-250 (Sigma)

Stock solution of 1.0g.l^{-1} CBBR-250 in 50% methyl alcohol.
For working solution, 10ml acetic acid added to 50ml stock solution and added to 40ml distilled water.

Stained for 12hr.

Gels were destained in 20% methyl alcohol/7.5% acetic acid until background was clear.

2.B Visualisation of Proteins Bound to Nitrocellulose Paper (NCP):

NCP was soaked in 50% methyl alcohol/5% acetic acid for 20min; washed in distilled water (2min) and incubated in Amido Black working solution (10ml Sigma concentrate + 40ml distilled water) for 30min. NCP was destained in several washes of 7% acetic acid until the background was clear.

Appendix 3: ESTIMATION OF PROTEIN CONCENTRATIONS:

3.A Folin - Ciocalteu Reaction; the Lowry Protein Assay:

The method is a slight modification of the procedure described by Lowry *et al* (1951).

Materials:

Folin Solution A: 2% (w/v) Na_2CO_3 in 0.1 N NaOH

Folin Solution B: 0.5% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 1% Na-K-tartrate

Folin-Ciocalteu Reagent: 10 vols Folin-Ciocalteu +
(diluted) 9 vols H_2O

Protein Standard: Bovine Serum Albumin Solution (1 mg/ml)

Preparation of Standard Curve:

The following were carried out in triplicate.

1. Into labelled, clean, dry test-tubes were pipetted 0, 0.05, 0.10, 0.15 and 0.20 ml of the protein standard solution.
2. The volume in each tube was made up to 0.6 ml with PBS. The Folin A and B solutions were mixed in ratio of 50:1, and 3 ml were added to each tube. The tubes were mixed well and left to stand for 10 at least 10 mins.
3. To each tube was added 0.3 ml of diluted Folin-Ciocalteu reagent. The tubes were mixed well and left to stand for 30 mins.
4. The optical density of the contents of each tube was read at 750 nm against a reagent blank (no protein).
5. A calibration curve, $E_{750 \text{ nm}}$ against protein concentration was plotted.

Estimation of protein content:

A range of dilutions of the protein sample was made. Into labelled test-tubes were pipetted 0.2 ml of each dilution. The volumes were each made up to 0.6 ml with water. Steps 2 to 4 inclusive were then followed as in the preparation of the standard curve.

3.B Bicinchoninic Acid Reaction; the Pierce BCA Protein Assay:

This method is derived from the standard procedure given in the BCA Protein Assay Handbook (Pierce).

Materials:

BCA Reagent A

BCA Reagent B

Protein Standard: Bovine Serum Albumin Solution (1mg.ml^{-1})

Preparation of Standard Curve:

The following were carried out in triplicate.

1. Into labelled, clean, dry test-tubes were pipetted 0, 0.05, 0.075, 0.10, 0.125 and 0.15ml of the protein standard solution.
2. The volume in each tube was made up to 0.15ml with PBS. BCA Reagents A and B were mixed together in a ratio of 50:1, and 3 ml were added to each tube. The tubes were mixed well and incubated at 37°C for 30 mins.
3. The optical density of the contents of each tube was read at 562nm against a reagent blank (no protein).
4. A calibration curve, $E_{562\text{ nm}}$ against protein concentration was plotted.

Estimation of protein content:

A range of dilutions of the protein sample was made. Into labelled test-tubes were pipetted 0.15ml of each dilution. Steps 2 and 3 were then followed as in the preparation of the standard curve, and the protein concentration of the sample was read off from the standard curve.